GENE EXPRESSION CHANGES IN HEIFERS TREATED MULTIPLE TIMES FOR BOVINE RESPIRATORY DISEASE

By

JAYMELYNN KAY JOHNSON

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RESPIRATORY DISEASE

Thesis Approved:

Dr. Clint Krehbiel

Thesis Adviser

Dr. Udaya DeSilva

Dr. Jerry Ritchey

Dr. Chris Richards

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER 1

INTRODUCTION

Cattle production is a large contributor to the economy of the United States. In 2007 the US cattle industry contributed $74 billion in retail sales. About half of this value ($36.1 billion) resulted from cattle and calf production alone. During 2008, 27.9 million steers and heifers and 5.8 million beef bulls and dairy cows were harvested, for a total harvest of 26.42 billion pounds of carcass weight. Of the total carcass weight, 5.4% was exported for a total of 1.1431 billion pounds and $2.175 billion profit (USDA website, 2008). Even though the beef industry is large, producers are looking for ways to produce the most economically efficient product due to small profit margins in cattle production. Therefore, it is necessary that producers obtain maximum body weight (**BW**) gain per unit feed cost. Research has been conducted to determine methods for increasing growth rate at decreasing cost. Producing cattle involves four equally important aspects that are required to produce the most efficient product. The four parameters include environment, health, genetics, and nutrition. Environment includes housing and/or pen conditions, and providing a clean supply of water. Health involves disease preventation measures such as vaccination and biosecurity as well as antibiotics when an animal becomes sick. Although genetics play a huge role in animal production efficiency, an animal's genetic potential may not be reached when environment and

health are compromised. A proper state of nutrition must be maintained in order to allow maximum expression of the genetic potential of an individual animal.

There is an intricate interrelationship between growth, nutrition, and immune status. In humans, inadequate diets result in poor appetite, malabsorption, and decreased growth and in turn this affects the consumption and absorption of nutrients that are critical for optimal immune response. The primary nutrients in immune function are zinc, selenium, vitamin A, pyridoxine, and vitamin E (Chandra, 1996). Results of human intervention trials indicate that modest supplements of micronutrients improve immune responses and more significantly, reduce the incidence of respiratory infection and antibiotic usage (Chandra, 1992). However, this has not been observed in many cattle trials. Cattle trials in which copper and zinc were injected or fed have evaluated humoral immune response. Increases in growth and production, have been observed, although there has not been a significant increase or change in immune response (Salyer et al., 2004, Nunnery et al., 2007). Carter et al. (2002), Rivera et al. (2003), and a review by Duff and Galyean (2007) reported some benefits to supplementing with vitamin E, although average daily gain (**ADG**), feed efficiency (**F:G**), and other performance measures were not increased. The authors suggested that further research is needed in regards to vitamin E and its effects on cattle immunological functions (Rivera et al., 2002).

Sick animals display a variety of clinical signs that can have a negative impact on cattle production efficiency. The primary cause for concern is poor appetite because animals that do not eat will not grow, negatively impacting ADG (Johnson, 1997; Smith, 1998) and immune function. Other common signs that are seen when an animal

experiences illness are anorexia (weight loss due to not eating), reduced locomoter activity, fever, motivational deficits, and inactivity, to name a few, and these also negatively impact animal performance and growth (Kent et al., 1992; Broussard et al., 2001; Dantzer et al., 2001). According to Dantzer et al. (2001) these changes in behavior are caused by a natural reorganization of the animal's priorities to cope with illness.

Klasing (1998) stated that the acute phase response is a process of nutrient liberation through skeletal muscle catabolism and nutrient consumption for acute phase protein synthesis and fever. With this increase in hepatic demand for amino acids to support gluconeogenesis and the acute phase response, there is a considerably greater need for amino acids to support the immune response (Klasing and Barnes, 1988; Laurin and Klasing, 1990; Koh et al., 1996; Klasing, 1998). This shift in priority for amino acid metabolism could also decrease growth of an animal.

The cattle industry is very diverse and complex with many segments used to produce one product. The first segment of the cattle industry is cow-calf operations. After weaning, calves may either go to a backgrounding/stocker operation that grows light weight calves to heavier weights before sending them to the feedlot, or the heavier calves may go directly to the feedlot. Because most cow-calf operations are located in areas that are not around feedlots, these cattle must be shipped to areas where feedstuffs and feedyards are prevalent. In a survey done by the USDA's APHIS, approximately 75% of cow-calf operators sell their calves through auction markets (summarized by Van Metre et al., 2009). Due to most of the cattle in the industry coming from small herds, they are commingled at weaning time to fill truckloads. In the process of going through weaning and commingling, these cattle get stressed causing higher incidences of disease

because the immune system becomes compromised. Shipping fever is a common disease that cattle get during this time. Indeed, shipping fever or bovine respiratory disease **(BRD)** is the primary disease causing the greatest economic loss to the cattle industry. The following review focuses on factors involved with, and the consequences of BRD.

CHAPTER 2

LITERATURE REVIEW

Respiratory infections are relatively common in agricultural animals and are a major economic burden because they inhibit weight gain and somatic growth (Escobar et al., 2002). Virtala et al. (1996) observed that body weight (**BW**) gain was decreased during the first month of life when calves were experiencing pneumonia. Over the length of the trial they observed that for each week of pneumonia total BW gain was decreased by 0.8 kg (Virtala et al., 1996).

Bovine respiratory disease is the most costly beef cattle disease in the U.S. Costs associated with BRD prevention, treatment, morbidity, and mortality in groups of cattle have been estimated from $13.90 (Snowder et al., 2006) to $15.57 (Faber et al., 1999) per animal. Annual losses to the U.S. cattle industry are estimated to approach $1 billion, whereas preventative and treatment costs are over $3 billion annually (Griffin, 1997). Snowder et al. (2006) reviewed health records for 18,112 over a 15 year period and observed that there was approximately a 17% incidence of BRD with a year percentage low of 5% and a high of 44%. Many times health technicians or "pen riders" diagnose BRD when it could be another form of respiratory disease. Common clinical symptoms for BRD include fever, rapid breathing, repetitive coughing, nasal or eye discharge or both, diarrhea, dehydration, and appetite depression. These symptoms

are similar if not identical to most forms of respiratory disease. Because diagnosing all respiratory problems as BRD is common in the industry, Snowder et al. (2007) used all symptoms that could have been BRD in which the majority of the classifications were for pneumonia and respiratory disease (86 and 11%, respectively). Additional disease classifications included for BRD were bronchitis, emphysema, pleuritis, pulmonary adenomatosis, upper respiratory infection, and pleural fibrosis (Snowder et al., 2007). If these numbers are indicative of the whole cattle population in the U.S., then there is a significant incidence of BRD within the feedlot industry.

Bacterial pathogens implicated in acute and chronic BRD include *Mannheimia haemolytica, Pasteurella multocida*, *Haemophilus somnus* and *Mycoplasma* spp. The major bacterial pathogen involved in acute BRD appears to be *Mannheimia haemolytica*, a normal commensal microorganism present in the bovine upper respiratory tract (Hodgson et al., 2005). There are also four viral pathogens that are associated with BRD: bovine herpes-virus type-1 (BHV-1), parainfluenza type-3 virus (PI_3V), bovine viral-diarrhea virus type-1 (BVDV), and bovine respiratory syncytial virus (BRSV) (Peters et al., 2004). Feedlots typically vaccinate against BHV-1-IBR-PI_3-BRSV-BVDV (5-way viral) as part of routine processing. These vaccines can be administered by either intranasal or intramuscular routes (Duff et al., 2000).

Bovine respiratory disease can be seen at many different stages throughout an animal's life. Within the feedlot there seems to be a greater prevalence of the disease in the early to mid phases of finishing. When animals are exposed to the virus or bacteria early in the feeding period they often experience a greater loss in carcass characteristics and ADG. Snowder et al. (2007) noted that cattle that were treated for BRD during the

late period of feeding tended to have heavier carcasses, more retail product, less fat trim, and heavier bone weight than cattle that were treated for BRD at the early and mid periods. They also noted that there was a decrease in external and internal measures of fat due to BRD, which is consistent with other reported data (Gardner et al., 1999; Roeber et al., 2001). There are differing results on the impact of BRD on carcass quality. Snowder et al. (2007) and Gardner et al. (1999) did not observe a significant decrease in marbling score in cattle treated for BRD, although Roeber et al. (2001) and Montgomery et al. (2009) observed significant decreases in marbling score.

Cystic fibrosis **(CF)** is a disease in humans that is caused by a genetic defect that impairs the mucous lining of the lungs. This predisposes patients to chronic bacterial infection in the respiratory tract that causes lung destruction and loss of pulmonary function. In CF the loss of lung function accounts for most of the morbidity and over 90% of the mortality (Elborn et al., 1991; Davis et al., 1996; Ionescu et al., 2002). Cystic fibrosis studies suggest that the host inflammatory and metabolic responses to chronic pulmonary infection have an impact on maintenance of body composition (Pencharz et al., 1984; Stutts et al., 1986; Elborn et al., 1993; Bell et al., 1996; Bell et al., 2000; Ionescu et al., 2000; Ionescu et al., 2002). Changes in body composition are noted to possibly come from a negative energy balance resulting from an inadequate energy intake to meet energy demands. Studies in humans have shown that there is a 25 to 80% greater energy requirement for patients that had moderate to severe lung disease from CF (Pencharz et al., 1984; Ionescu et al., 2002). Ionescu et al. (2002) hypothesized that excessive energy expenditure in patients with CF could be caused by the increased energy cost of breathing due to altered pulmonary mechanics, catabolic intermediary

metabolism due to chronic pulmonary infection, as well as an acute phase inflammatory response with increased circulating immunoreactive tumor necrosis factor alpha **(TNF-α)**, interleukin-1 **(IL-1)**, interleukin-6 **(IL-6)**, and counter-regulatory hormones that regulate the catabolic response with mobilization of fat and skeletal muscle as alternative energy sources (Van der Poll and Sauerwein, 1993; Ionescu et al., 2002). Ionescu et al. (2002) observed, in clinically stable adults with CF and chronic *P. aeruginosa* infection of the lungs, that there was an excessive breakdown of both cellular and connective tissue protein, and this was correlated with the degree of impaired lung function and the systemic inflammatory response. The fat-free-mass and the reduced body mass were likely consequences of protein breakdown, and the authors concluded that these relationships parallel acute phase inflammatory and catabolic host responses. As with many other CF studies patients in the Ionescu et al. (2002) experiment were lipolytic. Lipolysis may be indicative of energy-wasting due to "futile cycling" of substrates between fat stores and the liver. Tumor necrosis factor-α, IL-1, and IL-6 stimulate liver lipogenesis and peripheral lipolysis and may have a role in the impaired use of stored lipids, which increases the utilization of protein as an alternative energy substrate (Ionescu et al., 2002). Potentially the effects of destruction of the lungs in CF patients could be similar to what is seen in chronic BRD infections in cattle.

Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (**BVDV**) causes a complex of disease problems including respiratory disease, infertility and fetal infection. Fetal infection can lead to early embryonic death, abortion, congenital defects, stunting, or the birth of persistently infected (**PI**) calves. Persistently infected calves come from in utero exposure to the

noncytopathic biotype of BVDV by about 120 days of gestation. PI calves are continual shedders of the disease if they survive past infancy. These calves cause infection to other calves within their contemporary group. PI calves shed the virus through body secretions including nasal discharge, saliva, semen, urine, tears, milk, and feces (Rae et al., 1987; Brock et al., 1991; Bezek et al., 1995; Brock et al., 1998; Larson, 2007). There are two primary methods of transmission for BVDV; postnatal horizontal infection and gestational vertical infection. The way to test for a PI calf is to take ear notch samples for immunohistochemical testing. Decreased presence of PI animals near the herd or area will result in decreased prevalence of the disease. Bovine viral diarrhea virus is primarily a cow-calf issue; however, it does carry over into the growth phase. Ways to prevent this disease, or at least minimize it, are to vaccinate with BVDV vaccines in the unstressed, healthy heifer. These females can receive a modified live vaccine so that a protective immune response coincides with the first four months of gestation. In cows that have been previously vaccinated, it is recommended to give a shot once a year before the breeding season (Larson, 2007). Titers in colostrum from BVDV seropositive dams provide protection in young calves (Larson, 2005). Removing the seropositive animals from a herd as a biosecurity practice should help decrease the number of PI calves that will be produced. Testing all animals on a farm/ranch could help stop this disease, but most ranches do not routinely practice testing. One method to determine if BVD is prevalent in a herd is to review records and determine if a large proportion of abortions or open females exists. In stocker and feeder operations pregnancy is not an issue, but PI calves are the problem because they are the primary source of BVDV transmission to susceptible cattle during marketing, trucking, and while in feeding pens. Persistently

infected animals may have an impact on penmates and cattle in adjacent pens (Larson, 2005).

In the general cattle population the prevalence of PI cattle has been reported to be between 0.13% and 2.0% (Bolin et al., 1985; Howard et al., 1986; Wittum et al., 1990; Houe et al., 1995; Larson, 2007). Studies have reported pre-weaning mortality proportions of PI calves to be 20 to 83%, which can result in substantial loss in herds (Wittum et al., 2001; Larson, 2007), and economical loss for cow-calf producers. Other economic losses that can occur from BVDV are from PI calves shedding the disease to other healthy calves and compromising their immune system. Many times these diseases open the door for other respiratory diseases to cause illness and possibly death. Specifically, Martin et al. (1989), Fulton et al. (2000), and Fulton et al. (2002) observed that BVDV viremia and seroconversion can lead to respiratory disease outbreaks in feedlots. Decreasing the incidence of BRD and BVDV are two very important areas of research which could help beef cattle producers.

A Canadian immunohistochemical **(IHC)** and histological study looked at cattle that had chronic, antibiotic-resistant pneumonia, and sometimes concurrent polyarthiritis with infection of *Mycoplasma bovis* (*M. bovis*), BVDV, and *Haemophilus somnus* (*H. somnus*) (Shahriar et al., 2002). Shahriar et al. (2002) took 48 animal cases from feedlots during the years 1995 to 1998 that were submitted to the lab and tested for these pathogens. Shahriar et al. (2002) observed that *M. bovis* antigen was present in 44/48 cases and 15/16 cases in a follow-up study conducted in 1999. Bovine viral diarrhea virus was present in 31/48, and in the follow-up study 9/16 cases. Out of the 16 follow-

up cases four of them were types Ib and II BVDV. *H. somnus* antigen was present in the lung, heart, or both in 15/48 and 8/16 cases, respectively.

Pulmonary lesions were looked at and classified from the 48 cases into three groups; acute, subacute, or chronic (Shahriar et al., 2002). All stages of lesions were seen in all 48 cases. In acute or early lesions they observed a small amount of hypereosinophilic exudates within the bronchioles and terminal airways, but the epithelial lining was intact. The exudate was composed of degenerate neutrophils, macrophages, proteinaceous material, and necrotic debris and stained for *M. bovis*. In subacute lesions, there was focal to multifocal loss of bronchiolar epithelium. The bronchiolar lumen was dilated and filled with hypereosiophilic necrotic exudates. *M. bovis* was detected mainly in the necrotic exudates and at the periphery of the lumen. Most of the animals affected had their airways surrounded by lymphocytes and plasma cells or by large multifocal lymphoid aggregations. In chronic lesions, there were multifocal, well-circumscribed foci of coagulative or liquefactive necrosis, with eosinophilic debris. There was also degenerate neutrophils, cellular debris, and pyknotic nuclei, macrophages, and plasma cells mixed with fibroblasts. *M. bovis* antigen was present at the margins of the necrotic foci and extended into the mononuclear cell layer.

Shahriar et al. (2002) showed that mycoplasmal infection, particularly *M. bovis*, is common in chronic pneumonia of feedlot cattle and the organism is often isolated from the lungs. Due to the fact that >90% of the IHC results showed *M.* bovis, Shahriar et al. (2002) suggested that *M. bovis* should be considered as a principal pathogen in chronic unresponsive pneumonia of feedlot cattle. Even though they did observe a large number of isolates of *M. bovis*, Shahriar et al. (2002) suggested there might be other strains of

Mycoplasma that they were not able to isolate. This is because the cattle used in this trial came from the chronic pens and had been treated many times with different antibiotics. Shahriar et al. (2002) also noted different lesions such as necrotizing mycocarditis, necrotizing tracheitis, and panniculitis, which had not been previously associated with *M. bovis*.

Bovine viral diarrhea virus was observed in 64% of the 48 animals and 56% of the 16 animals, indicating a synergism between BVDV and *M. bovis*. Shahriar et al. (2002) was certain that the BVDV they observed was a primary infection and not PI calves. Bovine viral diarrhea virus infection is primarily responsible for vascular lesions observed primarily in the heart, but also in other tissues. There is not a consistent correlation between the presence of vascular lesions and antigen observed in animals that have BVDV. Incidence of vascular lesions can be affected by factors such as the stage of viral infection or immune status of the animal, as well as mechanisms associated with BVDV vasculitis (Shahriar et al., 2002).

Nutrition

Nutritionally deprived animals lose BW as evident by the study of Lehnert et al. (2006). Microarray and real-time polymerase chain reaction **(RT-PCR)** analyses were conducted on biopsy samples from cattle that had been nutritionally deprived for 114 days. Lehnert et al. (2006) observed that there are several crucial aspects of BW loss-mediated muscle atrophy, all of which may contribute to the ability to cope with and recover from periods of nutritional deprivation. Specifically bovine muscles preferentially sacrifice fast, glycolytic muscle fibers during BW loss conditions while showing a down-regulation of gene expression specific to fast-twitch fibers. There also

was a 2 to 6-fold decrease in expression of genes from the classes of muscle structural proteins, muscle metabolic enzymes, and extracellular matrix when compared to animals on a rapid growth diet. Lehnert et al. (2006) observed down-regulated genes in muscle with roles in myogenic differentiation, maintenance of mesenchymal stem cells, modulation of membrane function, prevention of oxidative damage, and regulation of muscle protein degradation.

Weight loss and manipulation of fatty acid profiles were also observed by Lehnert et al. (2006) with a significant increase in stearoyl-CoA desaturase gene expression observed in atrophying muscle, suggesting that increased fatty acid synthesis is part of the response to caloric restriction. This study also uncovered evidence for possible growth-factor-mediated maintenance of stem cell compartments during atrophy as well as possible activation of antioxidative mechanisms (Lehnert et al., 2006).

Muscle, Fat, Growth, and the Immune/Hormone Interactions

A new area of research in regard to animal growth and performance is the effect that an immunological challenge has on muscle and fat cells. These tissues are currently being considered as active secretory organs involved with immune response. Traditionally, adipose tissue was thought to be deposited on animals for providing excess energy stores. However, a more recent view of adipose tissue is that of an active secretory organ, sending out and responding to signals that modulate appetite, energy expenditure, insulin sensitivity, endocrine and reproductive systems, bone metabolism, inflammation and immunity (Fantuzzi, 2005). Adipose tissue produces and releases a variety of proinflammatory and anti-inflammatory factors, including the adipokines, leptin, adiponectin, resistin, and visfatin, as well as cytokines and chemokines such as

TNF-α, IL-6, monocyte chemoattractant protein 1, and others. It has been shown that a decrease in leptin levels might predispose an individual to increased susceptibility to infection caused by reduced T-cell responses in malnourished individuals (Fantuzzi, 2005). Adipose tissue can be divided into two major types: white adipose tissue and brown adipose tissue. White adipose tissue represents the vast majority of adipose tissue in the organism and is the site of energy storage, whereas brown adipose tissue is used for nonshivering thermogenesis. White adipose tissue cells are composed of many cell types (Curat et al., 2004; Fantuzzi, 2005).

Muscle cells have been shown to express receptors for both cytokines and growth factors (Broussard et al., 2003). Escobar et al. (2004) observed in studies with young swine infected with Porcine Reproductive & Respiratory Syndrome **(PRRS**; ATCC VR-2385 isolate P-129 containing 50% tissue culture infected dose**)** and *M. haemolytica* (strain P5722-3, broth containing 10^{10} color-changing units/L) that increases in inflammatory cytokines increase myostatin concentrations. Myostatin has been reported to inhibit growth and utilization of nutrients by growing muscle. Escobar et al. (2004) suggested that the magnitude of increases in inflammatory cytokines during acute respiratory infection may be predictive of decreases in protein accretion and growth, some of which is due to increases in myostatin. Escobar et al. (2004) hypothesized that these mechanisms could explain decreased weight gain and linear growth of young animals and human children. Cytokines have been shown to act on skeletal muscle to reduce the efficacy of anabolic hormones such as IGF-I and insulin, while also directly influencing the synthesis and degradation of skeletal muscle protein (Broussard et al., 2001; Alvarez et al., 2002; Escobar et al., 2004). Skeletal muscle may be an important

source of both catabolic and anti-inflammatory cytokines and the balance between these could possibly participate in the modulation of protein turnover and muscle wasting (Alvarez et al., 2002).

Recent evidence indicates that adipocytes and myofibers are equipped with functional pattern recognition receptors, and are capable of responding directly to pathogens and other receptor ligands. Adipocytes and myofibers, acting as functional pattern recognition receptors, are active participants in the innate immune response producing a number of immune and metabolic regulators, including pro-inflammatory cytokines and adiponectin which help in regulating homeostasis (Mohamed-Ali et al., 1998; Havel, 2002; Ding et al., 2004; Gabler and Spurlock, 2007).

The immune system does not function independently from other physiological systems, but is highly integrated with normal metabolism and physiology (Klasing, 1998). During the past 10 years, receptors for a number of protein hormones and cytokines from the immune system have been shown to use common intracellular messenger pathways. Cytokines and hormones act in a similar fashion and in some instances there has been an overlap at site receptors, especially because cells of the immune system have a wide variety of receptors, some of which use hormones that are normally regulated by the diet (Klasing, 1998). The use of common messengers at least partially explains some of the redundancies between hormones and cytokines (Broussard et al., 2003). Proinflammatory cytokines that are released following a pathological insult can act on the periphery, on the gastrointestinal tract, and in the brain to reduce food consumption (Broussard et al., 2001). Lipopolysaccharide (**LPS**) stimulates cytokine mRNA and protein expression in classical immune tissues such as the liver, spleen, and

lung, as well as non-immune tissues such as cardiac muscle (Frantz et al., 1999; Cowan et al., 2001).

The emerging idea is that cytokines from the immune system use intracellular substrates that are also regulated by hormone receptors, and this regulation is likely to control cytokine and hormone specificity and redundancy. This concept is consistent with the original ideas of Blalock (1984), in which both hormones and cytokines serve important messenger roles in immune-endocrine communication (Broussard et al., 2003). The possibility has only recently been considered, however, that hormones and cytokines might regulate the function of each other by using common intracellular substrates. For example, there is now accumulating evidence that receptors for some cytokines, such as IL-2, IL-4, IL-9, and interferon-γ, use intracellular docking molecules that were first identified for the insulin receptor (White, 1998; Broussard et al., 2003). This implies that a single cell can express receptors for both a hormone and a cytokine. Simultaneous activation of the cytokine receptor leads to downstream events that regulate the biological activity of the hormone receptor, and vice versa. Cytokines are considered to be the critical intermediates that regulate and coordinate activities between the immune and neuroendocrine systems (Broussard et al., 2003).

In regards to an interaction with the immune system and common growth promotants, most types of leukocytes express receptors for IGF-I, including T cells and their subsets, B cells, macrophages and neutrophils (reviewed in Kelley et al., 1992; Johnson, 1997; Kelley et al., 1998; Burgess et al., 1999). Growth hormone, as well as IGF-I, are potent signals for priming pig (Edwards et al., 1988; Fu et al., 1991), cattle (Fu et al., 1992), rat (Edwards et al., 1988, 1992), and human (Fu et al., 1991, 1992)

phagocytic myeloid cells for enhanced secretion of superoxide anion (Broussard et al., 2001).

The Immune System

The elimination of pathogenic microorganisms is efficiently achieved by two branches of the immune system: "innate immunity" and "adaptive immunity". Adaptive immunity includes two main classes of specialized B and T cells that recognize non-self antigens via unique receptors on their surface. Innate immunity appears early during evolution and has the ability to recognize pathogenic microorganisms by germ-line encoded receptors with defined specificities for highly conserved structures present on most pathogenic microorganisms. Innate immunity is activated immediately after infection and rapidly controls microorganisms until adaptive immunity takes over (Toubi and Shoenfeld, 2004).

Cytokines and Receptors in Immunity and Growth

Cytokines play an integral role in the immune response and may adopt both inflammatory and anti-inflammatory roles. Cytokines can function either locally in a paracrine and autocrine manner or at sites distant from the site of production in a manner comparable to the endocrine hormones. Based on the review article of Gabler and Spurlock (2007) there are thirteen genes that act in both growth and immune responses. These genes are TNF-α, nuclear factor kappa B **(NF-κB)**, IL-6, toll-like receptor 4 **(TLR-4)**, interferon gamma **(IFNγ)**, interleukin-15 **(IL-15)**, adiponectin, AdipoR1, AdipoR2, 5'AMP activated protein kinase **(AMPK)**, peroxisome proliferator-activated receptor alpha **(PPARα)**, glucose transporter 4 **(GLUT 4)**, and insulin growth factor one **(IGF-I)**. Many of these genes have been shown to be involved in growth and immunity in swine

and rats. Of these, the present project focuses on TLR-4, NF-κB, TNF-α, and IL-6 gene expression in cattle.

Toll-like Receptor 4

Toll-like receptor-4 is part of a large family of receptors that recognize pathogen-associated molecular patterns (Frost et al., 2002). TLRs are large sensors of microbial infection and are responsible for the induction of both innate and adaptive immune responses. The mammalian TLR family presently consists of ten members, and because of all the many receptors, it provides the immune system with the ability to respond to a wide variety of pathogens. Toll-like receptor-4 signaling can stimulate IL-6 mRNA expression in mouse skeletal muscle. Frost et al. (2003) observed that mice that have a mutation in the TLR-4 gene have a greatly reduced expression of IL-6. This could handicap an immune response and the ability to overcome an immunological challenge. TLR-mediated functions can be modulated by viral infection or activation of macrophages via IFN-γ; however, Franchini et al. (2006) showed that co-stimulation with IFN-γ or pre-infection with BVDV did not seem to change the concentrations of TLR mRNA transcripts. In contrast, TLR-mediated TNF and nitric oxide **(NO)** production are affected by TLR-4, TLR-2, and TLR-3. There is speculation that TLR-2 and TLR-4 are designed to recognize extracellular pathogen-associated molecular patterns **(PAMPs)**, whereas TLR-3 and TLR-9 are better positioned to recognize intracellular (viral) PAMPs (Franchini et al., 2006). Franchini et al. (2006) concluded that both TLR-2 and TLR-4 agonists induce NO synthesis and TNF expression of bovine macrophages and that NO synthesis is strongly enhanced by the presence of IFN-γ or pre-infection with non-

cytopathic BVDV. This confirms modulation of post-TLR-agonist-binding events by cytokines and infectious agents (Franchini et al., 2006).

Several reports emphasize that TLR-2 and TLR-4 can recognize a number of self protein (**HSP**) families. Self protein families such as HSP60 and HSP70 are normally intracellular proteins, but can also become "danger" molecules when released under stress or following excessive apoptosis. They activate vascular smooth muscle cells and macrophages via TLR-2 and TLR-4 to secrete pro-inflammatory cytokines such as TNF-α and IL-12, and to over-express co-stimulatory molecules on antigen presenting cells (Smiley et al., 2001; Bulut et al., 2002; Toubi and Shoenfeld, 2004). Toll-like receptor-4 along with TLR-1 and TLR-2 are expressed in immature dendritic cells. The expression of TLRs is modulated by microbial components such as LPS, CpG-DNA, microbacteria, and by Th1 cytokines such as IL-2, IFN-γ and TNF-α (Supajatura et al., 2001; Mellman and Steinman, 2001; Toubi and Shoenfeld, 2004). The TLR family is highly homogenous with the IL-1R family. Both receptors generate signals via homophilic interactions that when activated causes MyD88 to recruit the death domain, IL-1R-associated kinase (**IRAK**). There are four mammalian IRAK family members of which IRAK-4 regulates TLR 2, 3, 4, and 9. When IRAK-4 is absent, there is impairment of cytokine production, including TNF-α, IL-6, and IFN-γ (Toubi and Shoenfeld, 2004). MyD88 is an adaptor molecule that leads to the activation of NF-κB. Nuclear factor kappa B is the best defined transcription factor that is activated by TLRs (Toubi and Shoenfeld, 2004). Toll-like receptor-mediated activation induces the expression of co-stimulatory molecules such as CD80/86, and the production of IL-12, thus enabling efficient major histocompatibility complex (**MHC**) class-II-antigen presentation to naïve

T cells and the generation of an antigen specific adaptive immune response. The specific adaptive immune response is mediated by either Th1 or Th2, subject to the differential induction of variable TLRs by MyD88. In MyD88-deficient mice the production of IFN-γ from CD4+ T cells was impaired following their exposure to various antigens, suggesting that Th1 immune response is regulated by MyD88 signaling pathways (Kaisho et al., 2002; Toubi and Shoenfeld, 2004). Toll-like receptor-4 and TLR-2 on macrophages are triggered when a cell dies to clear up apoptotic bodies, maintaining self-tolerance and preventing tissue damage (Toubi and Shoenfeld, 2004).

Eriksson et al. (2003) used non-transgenic mice as a model where dendritic cells were loaded with heart-specific self-peptide induced CD4+ T-cell-mediated myocarditis. Toll-like receptor stimulation triggered self-peptide-loaded dendric cells which were shown to be required for disease induction (Eriksson et al., 2003). During remission from acute myocarditis, TLR re-stimulation resulted in a relapse of inflammatory infiltrates. It was suggested that this demonstrates how tissue damage and continuous activation of TLRs can provoke autoimmunity during chronic infection (Erikkson et al., 2003; Toubi and Shoenfeld, 2004). Toll-like receptor-4 recognizes LPS, which is unique to gram-negative bacteria. This recognition also requires a second polypeptide, MD-2, which enhances LPS responsiveness by binding to the extracellular arm of TLR-4 (Toubi and Shoenfeld, 2004).

Interferon γ and TNF-α individually induce modest levels of inducible nitric oxide synthase (**iNOS**) mRNA and NO and have a small effect on MyoD mRNA expression. Di Marco et al. (2005) concluded that the iNOS gene could be a common target activated synergistically by cytokines to trigger muscle fiber loss. In mice, both *in vivo* and *in vitro*

experiments have shown that these cytokines work in the above mentioned pathway (Di Marco et al., 2005).

Nuclear Factor Kappa B

Nuclear factor kappa B binds to the promoters of many genes involved in the immune response. Nuclear factor kappa B can be activated by LPS and IL-1 that are part of a large family of TLRs, and the first step is the proteolytic degradation of its inhibitory protein IκB by the proteasome (Frost et al., 2002). Nuclear factor kappa B belongs to a conserved family of proteins that form multiple homodimers and heterodimers with transcriptional activity (Ghosh et al., 1998). In most cell types, NF-κB dimers are retained in the cytoplasm by their interaction with specific inhibitors known as IκBs. The recognition of pathogen-associated molecular patterns such as double-stranded RNA, single-stranded RNA, lipopolysaccharide, or CpG oligonucleotides by membrane-associated receptors (TLRs) and cytoplasmic sensors leads to a series of events resulting in the ubiquitination and proteosomal degradation of the IκB subunit. NF-κB is then released and translocates to the nucleus, activating the transcription of genes involved in innate and adaptive immunity (Langland et al., 2006; de los Santos et al., 2007; Takeuchi and Akira, 2007). Frost et al. (2002) noted that NF-κB plays a role in regulating the TNF-α promoter in C2C12 muscle cells. They observed that LPS decreased IκB, -α, and -ε levels in C2C12 cells, and this was prevented by pretreatment with MG-132, which is a proteasomal inhibitor. Nuclear factor-kappa B has also been shown to induce the expression of IL-1β, TNF-α, and IL-8 in bovine alveolar macrophages exposed to *M. haemolytica* leukotoxin and endotoxin (Hsuan et al., 1999; Caverly et al., 2003). This was confirmed *in vivo* at Iowa State University, where Caverly et al. (2003) took

colostrum-deprived, Holstein steer calves and induced them with *M. haemolytica* by fiberoptic bronchoscopy. Their results showed that tracheal antimicrobial peptide (**TAP**) and IL-8 mRNA expression correlated positively with each other. In addition there was an increased translocation of NF-κB to the nuclei of epithelial cells in calves with *M. haemolytica* pneumonia (Caverly et al., 2003).

Viral activation of NF-κB is reflected by an increase in the expression of multiple factors involved in the innate and adaptive immune response, including the proinflammatory cytokine TNF-α and the chemokine RANTES (Collart et al., 1990; Shakhov et al., 1990; Moriuchi et al., 1997; Pahl, 1999; de los Santos et al., 2007). NF-κB is also activated by bacterial cell wall and viral products, mitogens, and reactive oxygen species (Baeuerle and Baltimore, 1996; Rothwarf and Karin, 2000). NF-κB has an integral role in innate immunity, inflammation, and apoptosis; however, its *in vivo* functions in muscle are unknown. NF-κB is a central integration site for proinflammatory signals and a master regulator of related target genes which include cytokines and chemokines, immune-presenting receptors, antigen-presenting receptors, stress response and acute phase proteins, and other regulators of apoptosis, growth, and host defense (Cai et al., 2004). In contrast to NF-κB being involved in muscle wasting with several other muscle wasting cytokines, Cai et al. (2004) observed via quantitative RT-PCR analyses normal muscle mRNA levels of NF-κB as well as normal circulating concentrations of TNF-α, IL-6, IL-1β, IFN-γ, IL-2, IL-8, IL-10, leukemia inhibitory factor (**LIF**), ciliary neuronotrophic factor (**CNTF**) and their respective receptors in MIKK mice muscle cells. MIKK mice have muscle specific expression of IKK (IκB kinase) which liberates NF-κB and allows translocation into the nucleus. Based on

normal cytokine muscle expression, Cai et al. (2004) suggested that NF-κB acts downstream of cachexia-inducing factors and that its activation is not sufficient to induce the transcription of cytokines reportedly linked to muscle wasting. This is a contradiction to NF-κB's function in inflammation and innate immunity where many cytokines activate NF-κB and more are released in a positive feedback response (Baeuerle and Baltimore, 1996; Van Antwerp et al., 1996; Delhase et al., 1999; Rothwarf and Karin, 2000; Cai et al., 2003).

Muscle atrophy has been considered to be primarily activated by NF-κB and stimulates the proteasome machinery by activating the muscle specific E3 ligase, MuRF1, leading to protein decay and muscle collapse. The NF-κB pathway also uses other activators that play a role in muscle degeneration/cachexia such as TNF-α and IFN-γ. Along with this, NF-κB expresses a wide variety of genes including those encoding cytokines, chemokines, adhesion molecules, and inducible effector enzymes (Ghosh and Karin, 2002). The iNos-NO system is used in conjunction with NF-κB in response to cachexia. Nitric oxide is an oxidative stressor in skeletal muscle of cachectic animals (Williams et al., 1994; Buck and Chojkier, 1996). Regulation of iNOS via the NF-κB pathway is important as it is used in a mechanism in the inflammatory process and is a potential target to combat inflammation-related disease (Di Marco et al., 2005). The iNOS gene transcription is thought to use NF-κB by the pathway where *de novo* transcribed message binds to HuR, which is an RNA-binding protein, and exports the message to sites for translation in the cytoplasm. This then induces iNOS protein synthesis which leads to the production and release of NO gas. Nitric oxide will likely

react with superoxide to form peroxynitrite, which ultimately mediates both MyoD mRNA decay and muscle atrophy (Di Marco et al., 2005).

Recent reports describe a role of NF-κB in cytokine-induced cachexia both in myotubes and in mice (Sandri et al., 2004). Starvation is also known to trigger this debilitating condition of cachexia (Cai et al., 2004; Sandri et al., 2004). However, based on the Di Marco et al. (2005) results cachexia and NF-κB are not related.

Tumor Necrosis Factor Alpha

TNF-α is known to be important in mediating inflammation and cytotoxic reactions. However, more recently it has been thought to have an active role in metabolism. Broussard et al. (2003) showed that when IGF-I receptors were stimulated by concentrations of lower than 0.1 ng/mL of TNF-α, it inhibited *de novo* protein synthesis by 50% in myoblasts. This interaction was suggested to provide the molecular basis for the cross-talk that exists for ligands in the immune and endocrine systems (Broussard et al., 2003). TNF-α as well as IL-1β and IL-6 are cytokines that are produced by macrophages in defense of an animal's immunological challenge. In addition to these cytokines' immunoregulatory role they also act on non-immune targets and have behavioral, endocrinological, and metabolic effects (Escobar et al., 2002). TNF-α has been shown in some cases, if it is chronically high, to result in muscle wasting in skeletal muscle due to protein turnover (Llovera et al., 1993). Tumor necrosis factor-alpha activates muscle protein degradation via the ubiquitin and ATP-dependent proteolytic pathway (Garcia-Martinez et al., 1993). Wasting disease in skeletal muscle has a role for TNF-α leading to cachexia (Argilés and López, 1999). Broussard et al.

(2001) showed that TNF-α can induce resistance of IGF-I at target tissues and can impair the biological activity of the receptor.

There are two isometric receptors for TNF-α that have been associated with cell death; p55 kDa (Type 1) and p75 kDa (Type 2). The TNF-α apoptosis signaling pathway also includes caspase-8 (FLICE)-induced activation of acid sphingomyelinase. Sphingomyelinase enzymatic activity results in cleavage of sphingomyelin to ceramide and phosphorylcholine (Schwandner et al., 1998). Ceramide produced by sphingomyelinase activates several pathways including the ceramide-activated protein kinase, ceramide-activated protein phosphatase, and phosphokinase C and these ceramide signals are known to promote apoptosis in various cell types (Mathias et al., 1998; Broussard et al., 2001). Frost et al. (1997) transiently exposed human myoblasts to TNF-α buffer and the myoblasts showed inhibition of protein synthesis. After removing the cytokine, TNF-α protein synthesis was still inhibited for greater than 48 hours. These observations suggest that a transient increase in plasma TNF-α concentration may impair protein synthesis long after the cytokine has disappeared from circulation (Frost et al., 1997; Cooney et al., 1999).

Skeletal muscle growth and regeneration requires fusion of progenitor mononucleated myoblasts into multinucleated terminally differentiated myofibers. Myoblasts fuse with myofibers, leading to incorporation of myoblast nuclei into myofibers (Novakofski and McCusker, 2001; Grounds, 2002). Insulin like growth factor-I increases key myogenic transcription factors that are required for myoblast fusion and induces specific protein synthesis such as myosin heavy chains (Arnold and Winter, 1998; Myer et al., 2001). Tumor necrosis factor-α has been proposed to act at the post-

receptor level to impair the ability of IGF-I to promote the synthesis of protein. Broussard et al. (2003) observed that tyrosine autophosphorylation of the alpha-chains of the IGF-I receptor was not affected by TNF-α. Instead, TNF-α inhibited the ability of IGF-I to tyrosine phosphorylate its two major docking proteins in myoblasts, insulin receptor substrate 1 and insulin receptor substrate 2 (Broussard et al., 2003). This slows down or stops the differentiation of muscle fibers.

TNF-α affects nearly every component of adipose tissue biology and therefore has a major role in regulating energy metabolism (Sethi and Hotamisligil, 1999). Systemically, TNF-α has been suggested to act in the brain to cause anorexia and subsequent BW loss (Oliff et al., 1987), and in a paracrine fashion, it has been suggested to limit adiposity by causing insulin resistance (Hotamisligil et al., 1993), inhibiting glucose transporter gene expression (Stephens and Pekala, 1991), and decreasing adipose tissue lipoprotein lipase activity (Beutler et al., 1985; Fried and Zechner, 1989; Mackay et al., 1990). TNF-α also alters lipid metabolism by stimulating lipolysis (Hardardottir et al., 1992), hepatic triglyceride synthesis, and subsequent hypertriglyceridemia (Adi et al., 1992). Finck and Johnson (2000) suggested that TNF-α modulates energy metabolism in ways that direct the animal towards a negative energy balance.

Interleukin-6

Interleukin-6 is an important pleiotrophic cytokine with both pro- and anti-inflammatory properties. Interleukin-6 is predominantly synthesized in macrophages, lymphocytes, liver, and spleen, but recently skeletal muscle is being recognized as a source of this cytokine. Interleukin-6 produced in the muscle has been said to have both local and systemic biological effects (Luo et al., 2003). An example of this is that IL-6

released from the muscle during exercise can stimulate lipolysis in adipose tissue and glycogenolysis in the liver (Pedersen et al., 2001; Luo et al., 2003). Acute-phase protein synthesis in the liver is regulated by IL-6 in which the primary contributor of IL-6 is from Kupffer cells; however, it is possible that IL-6 from muscle may contribute to the regulation of acute-phase protein production during inflammation. It is also possible that locally, IL-6 has trophic effects and may participate in tissue repair after injury and regeneration of muscle tissue in dystrophy and after denervation (Helgren et al., 1994; Kurek et al., 1996a,b; Luo et al., 2003).

In *in vitro* studies of C2C12 myoblasts, when LPS, IL-1β, or TNF-α were added IL-6 protein concentration increased by 6-to-8-fold and mRNA concentration by 5-to-10-fold (Frost et al., 2002). Frost et al. (2002) also observed that LPS stimulated IL-6 mRNA expression in both myoblasts and myotubes, but in regards to protein expression there was a greater increase in the myoblasts than the myotubes (Frost et al., 2002, 2003). Human and mouse myoblasts also express IL-6 in response to peptidoglycan from the cell wall of Gram-positive bacteria (Frost et al., 2003). When doing a trial *in vivo* in mice, Frost et al. (2003) observed that after injection of LPS plasma concentrations of IL-6 were increased. Blood was drawn from the mice at two, six, and 18 hours after injection to correspond with an early, middle, and late stage of the acute-phase response. During the early phase there was IL-6 detected at 2 ng/mL, with peak detection at six hours and was still significantly elevated during the late phase (18 hours). In mRNA extractions IL-6 was detected in skeletal muscle two hours after LPS administration which was 100-fold greater than that of the mice that received saline injections. At hour

six IL-6 was 20-fold greater than that of control mice but at hour 18 it returned back to baseline (Frost et al., 2003).

Muscle injury also increases IL-6 mRNA as detected by Kami and Senba (1998) who used *in situ* hybridization methods on mice tissues. Kami and Senba (1998) observed that IL-6 mRNA was significantly increased three hours post-trauma and the cells that expressed IL-6 were identified as mononucleated cells located in the extracellular spaces between damaged cells and in the damaged cells proper. This mRNA remained present in the tissue seven days post-trauma. No mRNA was detected in regenerating myotubes with centrally located myonuclei (Kami and Senba, 1998).

Luo et al. (2003) tested the hypothesis that IL-1β stimulates IL-6 production in cultured myotubes and tested whether mitogen-activated protein (**MAP**) kinase signaling and NF-κB activation are involved in IL-1β-induced IL-6 production. Luo et al. (2003) observed that treatment of cultured C2C12 cells for 24 hours with different concentrations of IL-1β showed a dose-dependent stimulation of IL-6 production with the max immune response seen at an IL-1β concentration of 1 ng/mL. To test whether the increased IL-6 concentration was associated with gene expression and mRNA levels, Luo et al. (2003) ran real-time PCR and saw a >25-fold increase in IL-6 mRNA expression. This confirms the fact that IL-6 production is controlled by IL-1β. Cytokine induced IL-6 synthesis is regulated by NF-κB in many cells, but in regards to skeletal muscle remains a mystery. Also, other studies have shown that the MAP kinase pathway mediates multiple responses to cytokine stimulation (Luo et al., 2003). To test this hypothesis, Luo et al. (2003) tranfected cells for NF-κB and also added inhibitors of the MAP kinase pathway to determine how they change the expression of IL-6 in the C2C12 myotubes.

They observed decreased expression of IL-6 in the cells when altering these pathways, thus concluding that NF-κB and the MAP kinase signaling pathway regulate the expression of IL-6 in IL-1β-stimulated myotubes (Luo et al., 2003).

Summary

There seems to be a gap in the understanding of how BRD directly impacts muscle, subcutaneous fat, and marbling, even though it has been well documented that sickness impacts growth and carcass quality. With greater incidences of clinical signs of BRD there are deceases in ADG, quality grade, ribeye area, and carcass weight. Decreased growth and carcass quality may result from inadequate intake during times of sickness, or it could be that gene expression and metabolism in economically important tissues are changing priorities. Instead of utilizing nutrients to increase in size, muscle and fat tissue might be repartitioning nutrients towards areas that have a higher demand, such as the immune response. This has been observed in human, mouse, and swine models, but not in cattle.

Chronic infections of BRD have not been well documented in terms of how the animals that survive perform. Most of the reports have been over the histological findings of the calves with "chronic pneumonia". Chronic cattle that survive still go into the food chain and need to be productive. Understanding the physiological changes that occur that negatively impact these cattle would be beneficial.

In human and mouse models, as well as *in vitro*, the acute phase cytokines have negative effects on the precursors to metabolism. These cytokines have been shown to be highly expressed in damaged tissues, during immune response, and by stimulation *in vitro*. These cytokines at appropriate levels are needed, but if over-expressed could be

causing excessive damage. For example, TNF-α can inhibit IGF-I and has been shown to cause muscle wasting, anorexia, and insulin resistance, each of which could slow growth and performance of cattle.

Because there is some understanding of how cytokines impact tissues *in vitro*, one purpose of the research presented herein was to determine how and where they function in cattle with chronic infection and after receiving multiple antimicrobial treatments. In addition, an understanding of changes in gene expression that potentially slow down adipose tissue and longissimus muscle growth is needed and was explored. Understanding how BRD and chronic muscle wasting impacts cattle production was the focus of this project.

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CHAPTER III

USE OF MICROARRAY TO DETERMINE GENES DIFFERENTIALLY EXPRESSED IN MUSCLE AND SUBCUTANEOUS FAT OF HEIFERS NEVER TREATED OR CONSIDERED CHRONICALLY MORBID AFTER A 63-DAY PRECONDITIONING PROGRAM

J. Johnson*, D. R. Stein*, L. O. Burciaga-Robles*, B. P. Holland*, D. L. Step†, C. J. Richards*, U. E. DeSilva*, and C. R. Krehbiel*,[1]

* Department of Animal Science, Oklahoma State Univesity, Stillwater 74078 and
† College of Veterinary Medicine, Oklahoma State University, Stillwater 74078

[1] Corresponding author: Dept. of Anim. Sci., 208 Animal Sci. Bldg., Stillwater, OK 74078; phone: 405/744-8856; fax: 405/744-7390; E-mail:clint.krehbiel@okstate.edu

Abstract: The objective was to determine the effects of bovine respiratory disease (BRD) on gene expression changes using microarray analysis in growing beef heifers. Tissue biopsy samples from the longissimus dorsi muscle (LM) and subcutaneous fat (SCF) between the 12th and 13th rib from heifers never treated against BRD (HLTH; n = 5) and heifers classified as chronically morbid (CHR; n = 5) were collected after a 63-d preconditioning period. Chronically morbid was defined as animals receiving at least three antimicrobial treatments and loss of BW during the previous 21 d on feed. Hybridizations were performed using a long oligo bovine array. Preprocessing and normalization of data was accomplished using the R-project statistical environment with

the Bioconductor and LIMMA packages through the GenePix AutoProcessor (GPAP 3.2). Significance level for differentially expressed genes was set at $P < 0.01$ with a twofold change or greater. Ontology analysis of the differentially expressed genes was carried out using GFINDer with emphasis on biological process and molecular function. To further elucidate the interaction(s) of annotated genes within the context of metabolic or signaling pathways, Ingenuity Pathways Analysis (IPA) was used to identify the most relevant biological mechanisms, pathways and functions of the differentially expressed genes. Of the 186 differentially expressed genes in LM (143 down- and 43 up-regulated) and the 121 differentially expressed genes in SCF (44 down- and 77 up-regulated), 146 and 97, respectively, had known ontology. Differentially expressed genes were mapped to pathways involved in immunological functions, metabolism, catalytic activities, binding, proteolysis, apoptosis, translation, transcription, growth, and transport of nutrients. These differences in gene expression across tissues and between treatment groups will provide a better understanding of the impact BRD has on immune response and animal growth.

Key words: animal growth, bovine respiratory disease, cattle, immune response, microarray

INTRODUCTION

Respiratory infections are relatively common in domestic livestock and are a major economic burden because they inhibit weight gain and somatic growth (Escobar et al., 2002). Virtala et al. (1996) observed that body weight (**BW**) gain was decreased

during the first month of life when calves were experiencing pneumonia. In addition, if calves were still experiencing pneumonia during the third month of life, for each week of clinical signs there was a 96 g decrease in average daily gain (**ADG),** and over the trial total BW gain was decreased by 0.8 kilograms (Virtala et al., 1996). Bovine respiratory disease (**BRD**) is the most costly disease for beef cattle producers in the U.S. Costs associated with BRD prevention, treatment, morbidity, and mortality have been estimated from $13.90 (Snowder et al., 2006) to $15.57 (Faber et al., 1999) per animal. Annual losses to the U.S. cattle industry are estimated to approach $1 billion, whereas preventative and treatment costs are over $3 billion annually (Griffin, 1997). Clinical symptoms for BRD include fever, rapid breathing, repetitive coughing, nasal or eye discharge or both, diarrhea, dehydration, and appetite depression (Snowder et al., 2007). In a 15-year data set with 18,112 feedlot cattle, Snowder et al. (2007) observed that there was approximately a 17% incidence of BRD with a year percentage low of 5% and a high of 44%. If these numbers are indicative of the whole cattle population in the U.S., then there is a significant incidence of BRD within the feedlot industry.

Bovine respiratory disease can occur at many stages throughout an animal's life. Within the feedlot there seems to be a greater incidence of the disease in the early to mid phases of finishing. When animals are exposed to one or a combination of viruses and bacteria early in the feeding period they often experience a greater loss in ADG and carcass quality (Snowder et al., 2007). A decrease in external and internal fat measures due to BRD has also been observed (Gardner et al., 1999; Roeber et al., 2001).

Cystic fibrosis (**CF**) is a disease in humans that is caused by a genetic defect that impairs the mucous lining of the lungs. This predisposes patients to chronic bacterial

infection in the respiratory tract that causes lung destruction and loss of pulmonary function. In CF the loss of lung function accounts for most of the morbidity and over 90% of the mortality (Elborn et al., 1991; Davis et al., 1996; Ionescu et al., 2002). In regards to CF, studies suggest that the host inflammatory and metabolic responses to chronic pulmonary infection have an impact on maintenance of body composition as summarized by Ionescu et al. (2002). Changes in body composition are noted to possibly result from a negative energy balance resulting from inadequate energy intake to meet energy demands. Studies in humans have shown that there is a 25 to 80% greater energy requirement for patients that had moderate to severe lung disease from CF (Pencharz et al., 1984; Ionescu et al., 2002). Ionescu et al. (2002) hypothesized that excessive energy expenditure in CF patients may result from the increased energy cost of breathing due to altered pulmonary mechanics, catabolic intermediary metabolism due to chronic pulmonary infection, or an acute phase inflammatory response.

Microarrays are a useful functional genomics tool to study the expression of genes at specific times in cells or tissues. Oligonucleotide microarrays are very useful especially in comparison to cDNA microarrays, because they can identify genes that are expressed in low abundance and are not represented in available cDNA libraries (Evans et al., 2008). Oligonucleotide probes have become the most popular format in most DNA microarray facilities (Kreil et al., 2006).

Because BRD is known to impact growth, performance, carcass quality, and profitability, we conducted an experiment to identify genes that are differentially expressed in economically important tissues in chronic vs. healthy heifers following a 63-d preconditioning period.

METHODS AND MATERIALS

The experimental protocol was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Animals

Three hundred sixty high-risk heifers (initial BW = 248 ± 14 kg) were purchased from multiple auction barns in Kentucky and delivered to the Willard Sparks Beef Research Feedlot (**WSBRF**), Stillwater, OK. The heifers were individually identified and ear-notched to test for persistent infection with bovine viral diarrhea virus (**BVDV**) as described by Fulton et al. (2006) in Kentucky before shipment. However, persistently infected heifers (n = 1) were not removed from the group until after arrival at the WSBRF. Upon arrival, heifers were weighed and given *ad libitum* access to prairie hay. Within 24 hours of arrival heifers were processed. Initial processing consisted of administration of a modified live viral respiratory vaccine (IBRV-BVDV Type 1 and 2-PI3V-BRSV, Pyramid 5, Fort Dodge Animal Health, Overland Park, KS), a 7-way Clostridial bacterin/toxoid (Vision 7, Intervet/Schering-Plough Animal Health, DeSoto, KS), and dewormed with a moxidectin (Cydectin, Fort Dodge Animal Health, Overland Park, KS). All heifers were implanted with estradiol and trenbalone acetate (Component TE-G, Elanco Animl Health, Indianapolis, IN). Seven days after initial processing heifers were re-vaccinated with Pyramid 5 (Fort Dodge Animal Health).

Once moved to assigned pens heifers were offered prairie hay and 1% of BW of a diet consisting of (DM basis) 45% dry rolled corn, 15% corn WDGS, 17.5% ground alfalfa hay, 17.5% ground grass hay, and 5% pelleted supplement [69.6% wheat middlings, 2% limestone, 7.5% salt, 2% magnesium oxide, 0.3% zinc sulfate, 0.14%

copper sulfate, 0.1% manganous oxide, 0.06% vitamin A (30,000 IU/g), 0.04% vitamin E (50%) and 0.25% Rumensin 80 (Elanco Animal Health, Indianapolis, IN)].

Heifers were observed daily for signs of BRD by trained professionals as described by Step et al. (2008). Briefly, evaluators used criteria based on the DART system (Pharmacia Upjohn Animal Health, Kalamazoo, MI) with modifications. The major observable signs were depression, appetite, respiratory signs, and rectal temperature. The heifers were assigned severity scores ranging from 1 to 4 where a 1 was assigned for mild symptoms, 2 for moderate, 3 for severe and 4 for moribund. The criterion for antimicrobial treatment was determined by using a combination of the visual observation ranking and rectal temperature. Animals that had a severity score of 1 and 2 had to have a rectal temperature of 40˚C or greater before an antimicrobial treatment was administered. If a heifer had a severity score of 3 or 4 she was automatically treated with an antimicrobial. The first antimicrobial treatment was tilmicosin (Micotil 300, Elanco Animal Health, Greenfield, IN) at a dosage rate of 10 mg/kg BW. If a second treatment was required (a minimum of 120 hours after first injection for severity scores of 1 or 2; 72 hours for 3 or 4), the antimicrobial used was enrofloxacin (Baytril 100, Bayer Corp, Shawnee Mission, KS) at a dosage rate of 10 mg/kg BW. If a third treatment was needed (at least 48 hours after second treatment) ceftiofur hydrochloride (Excenel RTU, Elanco Animal Health) was used at a dosage rate of 2.2 mg/kg BW. Forty-eight hours after the third treatment heifers were given another treatment with ceftiofur HCl. When visual and temperature criteria were not met, no antimicrobial was given, and both treated and non-treated heifers were returned to home pens following evaluation. Body weight was

recorded at the time of treatment and on d 63 to determine ADG during the preconditioning period.

After 63 days on feed, five heifers from the "healthy" (**HLTH**) group and five heifers from the "chronic" (**CHR**) group were chosen at random to be biopsied from the LM and SCF. Healthy was defined as an animal that had never been pulled for signs of BRD or any other disease and were never treated with an antimicrobial for clinical signs of BRD. Chronic was defined as heifers that had been identified as morbid and received antimicrobial treatment three times, were identified as being sick a fourth time, and lost BW during the previous 21 days. After the heifers were identified as CHR they were moved from their home pen to a chronic pen where heifers were placed on the growing diet described above and given free-choice prairie hay for the duration of the receiving period (total 63 days).

On d 63, biopsies were taken from the SCF and LM. After using a local anesthetic and cutting through the skin, thumb forceps were used to raise the SCF and a sample (approximately 100 mg) was removed with scissors. Biopsies for LM tissue followed the procedure described by Winterholler et al. (2008). Biopsy samples were immediately snap frozen in liquid nitrogen, and stored frozen (-80˚C) until further processing.

RNA extraction

RNA was extracted from both tissues using TRIzol following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, ~0.2 g of tissue was homogenized in 1 mL of TRIzol. The homogenate was clarified by centrifuging at 12,000 × *g* for 10 min at 4˚C. This removed the excess fat, leaving a purer aqueous phase of RNA, DNA, and

protein. Chloroform (0.2 mL) was added and incubated at room temperature for 10 min. Separation was done by centrifugation at 12,000 × *g* for 15 min at 4˚C. The aqueous phase (RNA) was removed and precipitated out with isopropanol. Ethanol (75%) was included to wash the pellet and then the pellets were dissolved in DEPC water.

A 1.5% agarose gel with ethidium bromide was used to determine the quality of the RNA. The quantity was determined using a Nanodrop™ ND-100 spectrophotometer (NanoDrop Technologies, Willington, DE).

Microarray

A long oligo, whole bovine array from the Bovine Microarray Consortium (Elsik, 2006 http://animalsciences.missouri.edu/animalgenomics/bomc.php) was used to randomly compare HLTH to CHR heifers. Total RNA (500 ng) was used in an EPICENTRE TargetAMP™ 1-round aminoallyl-aRNA amplification (EPICENTRE Biotechnologies, Madison, WI) protocol with a few modifications. In the in vitro transcription of aminoallyl-aRNA step, 6.0 µL of aminoallyl-UTP per reaction instead of 2.4 µL per reaction was added. Alexa Fluor™ 546 tagged the reference sample (HLTH) and Alexa Fluor™ 647 tagged the test sample (CHR).

Slides were pre-hybridized by being rinsed in 0.1% sodium dodecyl sulfate (**SDS**) for 5 min, rinsed in sterile double distilled water (**ddH2O**) for 15 s, and immediately dried for 2 min using a slide centrifuge. The slides were placed into a 50 mL conical vial that contained warmed (48˚C) commercial pre-hybridized buffer (BlockIT Microarray Blocking Solution, TeleChem Int., Sunnyvale, CA) and incubated at 48˚C for 4 h. The slides were then rinsed in sterile ddH2O for 15 s and dried for 2 min using a slide centrifuge.

The entire 80 µL hybridized sample with dye and buffer was placed under a 24 × 60 mm lifterslip (Erie Scientific, Portsmouth, NH) on the warmed (hybridized) slide (42˚C). The slides were placed into a hybridization chamber and maintained overnight (20 h) in a 42˚C hybridization oven. After hybridization the post-hybridization wash steps included disassembling the chamber by immersing the slide and cover slip into a 50 mL conical tube with 2X sodium chloride/sodium citrate (**SSC**)/0.2 SDS (42˚C). The slide was transferred to another conical with 2X SSC/0.2 SDS (42˚C) for 15 min with gentle agitation. It was then transferred to a 2X SSC wash for 15 min at room temperature with gentle agitation. The final wash was in a 0.2X SSC for 15 min at room temperature with gentle agitation. The slide was then spun dry for 1 min using a slide centrifuge.

Analysis of slides

The slides were scanned at two wavelengths (Alexa 555 and Alexa 647) using a ScanArray™ Express confocal laser scanner (PerkinsElmer Life Sciences Inc., Boston, MA) at a pixel size resolution of 10 microns with the resulting image saved as a 16 bit TIFF image. The image was placed into a commercial software package (GenePix™ Pro 5.0, Axon Instruments Inc., Union City, CA) used to analyze spot intensity while subtracting local background. The GenePix Pro results (GPR) were then placed into the GenePix AutoProcessor (GPAP 3.2) website from the Oklahoma State University Department of Biochemistry and Molecular Biology (http://darwin.biochem.Okstate.edu/gpap32/). The R-project statistical analysis was accomplished while using the Bioconductor and LIMMA packages where background correction was performed using Robust Multi-array Average (RMA) algorithm (Allison et al., 2006). A moderated T-test was used to identify the differentially expressed array elements along with a M value

(expression ratio = log2(647intensity/546intensity)) and *P*-value, obtained from the moderated t-statistic after false discovery rate using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Significance level for differentially expressed oligos was set at 't'>2 (absolute value), a M-value>+0.9 or <-0.9 (which represents a fold change of >1.87 or <-1.87), and a *P*-value < 0.01.

The significant gene sequences were placed into multiple different software programs to determine the ontology of the differentially expressed oligos; Entrez Gene (http://www.ncbi.nlm.nih.gov), Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/), and Genome Functional Integrated Discoverer (GFINDer) (http://www.medinfopoli.poli.it/GFINDer/).

Validation of microarray

Total RNA (1 μg) was used to make cDNA with the QuantiTect® Reverse Transcription kit following the manufacturer's protocol (QIAGEN Inc., Valencia, CA). The cDNA was then used as the template for real-time PCR. Ten genes were selected to be validated (twelve samples); three were up-regulated and three down-regulated in each tissue type with two genes that were differently expressed in both tissues (Table 1). The primers were designed using a sequence designed from Primer 3 (http://frodo.wi.mit.edu/) and Integrated DNA Technologies (IDT, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) software. The primer sequences, temperatures, and product size are described in Table 1.

An iCycler real-time detection system (Bio-Rad Laboratories, Inc., Herules, CA) was used along with SYBR Green Supermix to determine gene expression. 18S was used as the housekeeping gene. An efficiency curve for the reaction was done using five serial

dilutions. Thermal cycling conditions were 95˚C for 30 s, followed by 40 cycles of 95˚C for 15 s, variable annealing temperatures for 30 s, and 72˚C for 30 s. This was followed by a melt curve analysis by increasing the reaction to 95˚C for 1 min, 55˚C for 1 min, and then increasing the temperature by 0.5˚C from 55˚C to 94.5˚C. The samples were analyzed in triplicate.

Gene expression changes were calculated as described by Livak and Schmittgen (2001). A cycle threshold (C_T) was assigned to each of the samples. A ΔC_T value was calculated for each replicate by subtracting 18S rRNA C_T from the corresponding gene samples C_T. An average of the replicates was utilized to compare the gene expression levels to determine directionality. Fold change was calculated by determining the $\Delta\Delta C_T$. This was accomplished by subtracting the lowest expression level from the highest and plugged into the formula: fold change = $2^{-\Delta\Delta C_T}$.

Ingenuity Pathway Analysis

Ingenuity Pathways Analysis (**IPA**) (Ingenuity® Systems, http://www.ingenuity.com) was utilized to identify the most relevant biological mechanisms, pathways and functions of differentially expressed genes. IPA enables the visualization and exploration of gene interactions and relies on the most currently known relationships among human, mouse, and rat genes and proteins. All differentially expressed genes and M-values were imported into the program and the maps were utilized to determine functional relationships.

Statistics

The experiment was a completely randomized design. Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Results are discussed as significant if $P \leq 0.05$ and as tendencies if $P > 0.05$ to $P \leq 0.10$.

RESULTS

Biological data

To validate our experimental model treatment records and growth variables were recorded for each heifer. On average, day to first, second, and third antimicrobial treatment for chronic heifers was 2 ± 1.5, 8 ± 2.2, and 13 ± 5.3 from arrival, respectively; the range of treatment days was 0 to 23. The average day that heifers were moved to the chronic pen was 32 ± 13 days on feed. From d 0 to 63, ADG for HLTH heifers was 1.58 ± 0.59 kg/d, whereas ADG for CHR heifers was 0.61 ± 0.69 kg/d.

Microarray

Microarray results showed that there were 186 differentially expressed genes in the LM with 43 up-regulated and 143 down-regulated. Up-regulated indicates that the gene (spot intensity) was more highly expressed in CHR than HLTH. Down-regulated indicates that the gene was less highly expressed in CHR in relation to HLTH. In the SCF there were 121 differentially expressed genes; 77 were up-regulated and 44 down-regulated. Up-regulated genes were more prevalent in SCF, whereas the number of down-regulated genes was greater in LM.

After identifying the differently expressed oligos, sequences were run through the Basic Local Alignment Search Tool (BLAST) from the National Center for

Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligos were identified and given an accession code (Entrez gene ID) to determine ontology. Ontology consists of molecular function, biological process, and cellular components. For the purpose of this experiment we used the molecular function and biological process for general groupings of ontologies. Within the LM the largest percentage of differently expressed genes were in the metabolic process category, followed by binding, cellular process, and proteolysis (Table 2). In LM, chronic animals showed an increase in expression of genes that regulate protein modification/proteolysis. In SCF chronic animals had an increase in expression of genes that modulate transcription, fatty acid metabolism, multi-cellular processes, and apoptosis. In CHR animals there was a decrease in LM for genes involved in metabolic activities and in SCF for immunological function and translation. Also, within both tissues CHR heifers had a decrease in expression of genes involved in transport of nutrients and binding. In SCF the largest percentage of differentially expressed genes was in the binding and immunological category, followed by multicellular processes and proteolysis (Table 2). Grouped by ontology, Table 3 shows the individual differentially expressed genes in LM, and Table 4 shows the individual differentially expressed genes in SCF. There were two genes that were shown to be significantly differentially expressed in both tissues. Ribosomal protein S19 binding protein 1 (**RPS19BP1**) was down-regulated and Homeobox A5 (**HOXA5**) was up-regulated in both tissues.

RT-PCR

75% of the genes that were chosen for validation showed the same directional tendencies. Of the genes that were randomly chosen to validate the microarray three out

of the 12 showed opposite directional tendencies. Those genes were fatty acid binding protein 5 (**FABP5**), cathelicidin 5 (**CATHL5**), and RPS19BP1. All three of these genes were down-regulated in the microarray, FABP5 and RPS19BP1 in LM and CATHL5 in SCF. Figure 1 shows the genes that were validated and the directional tendencies of both the microarray and RT-PCR results.

In LM, fold expression of 26S tended to be greater ($P = 0.10$; Figure 2) in CHR heifers compared with HLTH heifers. Haptoglobin (**HP**) expression in SCF was greater ($P = 0.03$) for HLTH compared with CHR heifers (Figure 3). Although directional changes were similar to the microarray, differences among treatments were not observed ($P > 0.10$) for the remaining genes used for validation via RT-PCR.

DISCUSSION

In the present experiment, several genes involved in the ubiquitin pathway were up-regulated in chronic heifers (Figure 4). In human and mice experiments it has been shown that when the ubiquitin pathway is over-stimulated it leads to chronic muscle wasting and atrophy (Cao et al., 2005). Muscle is not only used in contractile functions, but when stress occurs protein stored in muscle is mobilized as a source of amino acids for energy (Cao et al., 2005). As summarized by Cao et al. (2005), the ubiquitin-proteosome pathway contributes to the majority of intracellular proteolysis in muscle. This system works by linking chains of polypeptide cofactor ubiquitins onto proteins to mark them for degradation by the 26S proteosome. The three enzymes in the chain are E1, E2, and E3. E1 are the ubiquitin-activating enzymes; E2 are the ubiquitin-conjugating enzymes, and E3 are the ubiquitin-protein ligases (Tisdale, 2007). There are

several dozen E2 in a cell and more than a thousand E3 (Cao et al., 2005). Multiple E3, which is the final step in the process that tags the protein for cleavage, allows for multiple routes by which this pathway can be activated and lead to muscle wasting. Tisdale (2007) suggested that the ubiquitin-protease pathway has three main E3 that are catabolically up-regulated in skeletal muscle in response to stress; muscle RING finger 1 (MuRF1), muscle atrophy F-box (MAFbx), also called atrogin, and E3α-II.

In the present experiment, genes that were up-regulated in LM involved in the ubiquitin-protease pathway were autocrine motility factor receptor (**AMFR**), Fas (**TNFRSF6**) associated factor 1 (**FAF1**), ankyrin repeat and SOCS box containing 2 (**ASB2**), 26S proteosome (**26S**), proteasome (prosome, macropain) 26S subunit, ATPase, 1 (**PSMC1**), and ATPases (both **ATP2B2** and **ATP8B2**) (Figure 5). These genes are connected in both function and within pathways that include heat shock protein 70 (**Hsp70**) and down-regulated genes toll interacting protein (**TOLLIP**) and hypoxia-induced factor 1, alpha subunit (basic helix-loop-helix transcription factor; **HIF1A**). Chiu et al. (2008) showed that AMFR exhibits E3 ligase activity which alters cellular adhesion, proliferation, motility, and apoptosis. In humans, AMFR has been observed to be expressed in numerous cancers primarily in the lung, esophagus, stomach, colon, rectum, liver, and skin (Chiu et al., 2008). FAF1 is a protein that has multi-ubiquitin-related domains and several different functions. FAF1 is a type of E3 protein and in humans acts as a tagging site for apoptosis and directly affects NF-κB activation through IκK cleavage (Song et al., 2005). FAF1 in humans acts as a binding protein for the ATPase domain Hsp70 that activates immunoprecipitation (Kim et al., 2005). The primary cleaving protein involved in the ubiquitin pathway, 26S proteosome, was also

up-regulated in LM in the present experiment (Figure 2). Changes in expression of several genes in the ubiquitin-protease pathway in CHR heifers in the present experiment may suggest that severe or chronic BRD may lead to muscle wasting and atrophy, which could have long-term negative effects on animal growth and carcass quality.

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1 (**NDUFA1**), an ubiquitione, is initially activated by hepatocyte nuclear factor 4, α **(HNF4A)**, which was shown through IPA analysis to increase expression of many genes that were differentially expressed in the array of the present experiment (Figure 6). ASB2 is predominantly expressed in muscle and if observed to be overexpressed results in inhibition of myoblast differentiation (McDaneld and Spurlock, 2008). ASB2, primarily the SOCs box, parallels F-box and WD repeat domain containing 12 (**FBXW12**; Hershko and Ciechanover, 1998). Other genes up-regulated involved with the ubiquitin pathway included RWD domain containing 1(**RWDD1**), sumo1 activating enzyme subunit 1 (**SAE1**), and ubiquitin specific peptidase 15 (**USP15**). FBXW12, RWDD1, and USP15 are ubiquitone E3, and SAE1 is an E1.

Hypoxia-induced factor 1, α subunit (**HIF1A**) stimulates angiogenesis which increases the establishment of new blood vessels. However, in humans it has been shown that HIF1A, when bound to von Hippel-Lindau (**VHF**), results in ubiquitin tagging and ultimate degradation (Petroski, 2008). HIF1A was down-regulated in LM in the present experiment.

In SCF, Ingenuity software revealed several up-regulated genes and down-regulated genes involved with HNF4A (Figure 6). Hepatocyte nuclear factor 4, α has been identified in several human diseases that stem from problems with the liver and

insulin resistance (Love-Gregory and Permutt, 2007). Diabetes and improper growth and development of the hepatic system occur with mutations in this transcription factor (Velho and Froguel, 1998; Maestro et al., 2007). Beta-estradiol was also a central hub within the SCF Ingenuity pathway that showed effects involving differentially expressed genes (Figure 6). Genes that were up-regulated included calponin 1, basic, smooth muscle (**CNN1**), and ankylosis, progressive homolog (**ANKH**), and down-regulated included chromosome 9 open reading frame 16 ortholog (**C11ORF10**) and wingless-type MMTV integration site family, member 11 **(WNT11)**. The down-regulation of CNN1 could be a potential trigger for the activities of HNF4A (Figure 6).

Genes that affect hepatic fibrosis and hepatic stellate cell **(HSC)** activation were also up-regulated and could be another potential cause of decreases in growth and performance of CHR animals. Hepatic stellate cells have a central role in hepatic fibrogenesis (Friedman, 2000). Hepatic fibrogenesis is scarring of the liver that can come from a variety of different factors like necrosis due to stress and lymphocyte activation. We observed up-regulation of collagens, **COL1A2** and **COL1A1** in LM and SCF in the present experiment. The collagens are affected by Smad (primarily Smad3) which leads to increases in both COL1A1 and COL1A2.

In conclusion, decreasing the detrimental effects of an over-activated ubiquitin-proteosome pathway could decrease the instances of muscle wasting in heifers treated multiple times for BRD. However, appropriate base-line expression levels should be determined along with potential side-effects of slowing this process due to the need for certain stages of transcription and cellular activation. In addition, greater growth rates in HLTH heifers could be due to the greater expression of genes that modulate metabolism

and immunological functions, and potential links between a pathogen insult and changes in gene expression should be further explored. Use of the whole genome microarray was a beneficial tool to look at differences in chronic animals versus animals that were considered to be healthy. This approach allowed us to identify pathways that when impacted could potentially decrease growth and carcass merit.

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Table 1: RT-PCR primers used in the validation step.

Gene	Tissue		Primer	Product Length	Anneal Temp.	Entrez ID
26S	LM	Forward	5'-CAGTGATGAAGGTGGAAAAGG-3'	184	59	506238
		Reverse	5'-GCGGACCATAGAGAATGACC-3'			
CADH1	SCF	Forward	5'-CGTTTCCTAAGTCGCTGGTC-3'	272	55.5	282637
		Reverse	5'-GGGCTTGTTGTCATTCTGGT-3'			
CATHL5	SCF	Forward	5'-AGGACGATGAGAACCCAAAC-3'	112	58	282167
		Reverse	5'-CCACACACTCTTTCAGCAGC-3'			
CXCL3	LM	Forward	5'-GCCAAACCGAAGTCATAGCC-3'	249	58	613667
		Reverse	5'-AAATAGTCCAGCACATCAAGTCC-3'			
FABP5	LM	Forward	5'-ATGGCTCTGCGAAAAGTGG-3'	145	59	281760
		Reverse	5'-GCTGTGGTCTCTTCAAACTTCTC-3'			
HOXA5	LM & SCF	Forward	5'-GACCTCGTTTAGTGCCAATG-3'	224	62-LM	768039
		Reverse	5'-CAGAGTCACAGTTTTCGTCACAG-3'		61-SCF	
HP	SCF	Forward	5'-CGTGTGGGTTATGTGTCTGG-3'	275	62	280692
		Reverse	5'-GTGTCGTCTTCCTTGTCGTG-3'			
ITGB8	SCF	Forward	5'-CAGTTTCACCATACATTAGCATCC-3'	244	58	3696
		Reverse	5'-AGCCTCTTTTCGCCATCC-3'			
RPS19BP1	LM & SCF	Forward	5'-CAGGAGAACCAGGAAGAAACC-3'	152	62-LM	509108
		Reverse	5'-TAGAACCCGAAGCCTACCC-3'		61-SCF	
TRA1	LM	Forward	5'-TCTTGCTGTGGTTTTGTTTG-3'	238	58	282646
		Reverse	5'-TTGTTCTTCCTCGTCTGTTCC-3'			

Table 2: Ontological groupings for LM and SCF.

Category	% LM[1]	% Up- LM[2]	% Down-LM[3]	% SCF[4]	% Up-SCF[5]	% Down-SCF[6]
Proteolysis	11.9	16.1	10.3	13.9	8.6	20.0
Apoptosis	2.8	6.5	1.3	1.5	2.9	--
Binding	14.7	6.5	17.9	18.5	17.1	20.0
Cellular processes	11.9	9.7	12.8	10.8	14.3	6.7
Translation	5.5	9.7	3.8	6.2	2.9	10.0
Transcription	7.3	6.5	7.7	4.6	8.6	--
Metabolism	25.7	22.6	26.9	9.2	11.4	6.7
Nutrient Transport	8.3	6.5	9.0	12.3	11.4	13.3
Multi-cellular process	0.9	3.2	--	4.6	8.6	--
Immune response	7.3	9.7	6.4	18.5	14.3	23.3
Structural component	3.7	3.2	3.8	--	--	--

[1]Percentage of differentially expressed genes within category in LM.
[2]Percentage of genes up-regulated in LM.
[3]Percentage of genes down-regulated in LM.
[4]Percentage of differentially expressed genes within category in SCF.
[5]Percentage of genes up-regulated in SCF.
[6]Percentage of genes down-regulated in SCF.

Table 3: Differentially expressed genes in LM grouped based on ontology – molecular function and biological process.

Entrez ID	Gene Name	M value
Proteolysis/protein modification/catabolism		
541216	RWDD1 RWD domain containing 1 [Bos taurus]	1.38
539244	ASB2 ankyrin repeat and SOCS box containing 2 [Bos taurus]	1.96
515109	TIMM8a translocase of inner mitochondrial membrane 8 homolog A (yeast) [Bos taurus]	-1.50
507222	TMEM208 transmembrane protein 208 [Bos taurus]	2.81
618428	CACYBP calcyclin binding protein [Bos taurus]	-2.89
505512	SAE1 sumo1 activating enzyme subunit 1 [Bos taurus]	2.23
404101	BMPR1A bone morphogenetic protein receptor, type IA [Bos taurus]	-1.22
506238	PSMC1 proteasome (prosome, macropain) 26S subunit, ATPase, 1 [Bos taurus]	3.51
510524	CTSH cathepsin H [Bos taurus]	-2.26
451586	UBE4A ubiquitination factor E4A (UFD2 homolog, yeast) [Pan troglodytes]	-1.95
789588	LOC789588 similar to chromosome 9 open reading frame 3 [Bos taurus]	-1.46
520170	UCHL3 ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase) [Bos taurus]	-2.10
518834	UBA6 ubiquitin-like modifier activating enzyme 6 [Bos taurus]	-1.76
Apoptosis		
282646	TRA1 tumor rejection antigen (gp96) 1 [Bos taurus]	-3.16
533406	PDCD6IP programmed cell death 6 interacting protein [Bos taurus]	1.28
531770	FAF1 Fas (TNFRSF6) associated factor 1 [Bos taurus]	3.43
Cellular process		
281950	NRXN1 neurexin 1 [Bos taurus]	-2.37
528265	SNX3 sorting nexin 3 [Bos taurus]	-1.21
281798	GNRHR gonadotropin-releasing hormone receptor [Bos taurus]	-1.21
511812	GNG11 guanine nucleotide binding protein (G protein), gamma 11 [Bos taurus]	-1.00
506708	RADIL Rap GTPase interactor [Bos taurus]	1.24
287018	GNG guanine nucleotide binding protein (G protein), gamma 5 [Bos taurus]	-1.65
532389	MRPL44 mitochondrial ribosomal protein L44 [Bos taurus]	-2.74
511080	CCDC11 coiled-coil domain containing 111 [Bos taurus]	-1.84
404159	TXNDC17 thioredoxin domain containing 17 [Bos taurus]	-2.21
504519	CAP1 CAP, adenylate cyclase-associated protein 1 (yeast) [Bos taurus]	1.42
287016	H2AFZ H2A histone family, member Z [Bos taurus]	-1.64
445425	TKT transketolase [Bos taurus]	4.02
280939	TEK TEK tyrosine kinase, endothelial [Bos taurus]	-1.59
Translation		
504754	MTRF1 mitochondrial translational release factor 1 [Bos taurus]	2.06
510729	TRPT1 tRNA phosphotransferase 1 [Bos taurus]	1.77
404190	RPL27A ribosomal protein L27a [Bos taurus]	2.06
527129	SRP9 signal recognition particle 9kDa [Bos taurus]	-1.29
615845	GATC glutamyl-tRNA (Gln) amidotransferase, subunit C homolog (bacterial) [Bos taurus]	-1.97
520875	EEF1B2 eukaryotic translation elongation factor 1 beta 2 [Bos taurus]	-0.99
Trascription		
782993	RPL7 ribosomal protein L7	-2.30
282711	EPAS1 endothelial PAS domain protein 1 [Bos taurus]	1.28
515561	ZNF462 zinc finger protein 462	-2.39
614212	LMO4 LIM domain only 4 [Bos taurus]	-2.14
540959	CRTC2 CREB regulated transcription coactivator 2 [Bos taurus]	-2.17
616523	SLMO2 slowmo homolog 2 (Drosophila) [Bos taurus]	1.35
513254	PEBP4 phosphatidylethanolamine-binding protein 4 [Bos taurus]	-1.70
508005	EXOSC9 exosome component 9 [Bos taurus]	-2.56
Metabolism		
613932	PLD3 phospholipase D family, member 3 [Bos taurus]	-2.33
281615	ALDH1A1 aldehyde dehydrogenase 1 family, member A1 [Bos taurus]	2.14
613800	ATP2B2 similar to plasma membrane calcium ATPase 2 [Bos taurus]	-2.09

512468	GCLC glutamate-cysteine ligase, catalytic subunit [Bos taurus]	-1.91
508712	CCBL2 cysteine conjugate-beta lyase 2 [Bos taurus]	-1.56
511901	LOC511901 similar to H1 histone family, member X [Bos taurus]	-2.38
281814	HIF1A hypoxia-induced factor 1, alpha subunit (basic helix-loop-helix transcription factor) [Bos taurus]	-1.96
541108	LOC541108 similar to histone cluster 2, H2aa4 [Bos taurus]	-1.52
507631	TYMS thymidylate synthetase [Bos taurus]	-2.21
508131	ZNF836 zinc finger protein 836 [Bos taurus]	-2.30
615692	SAP18 Sin3A-associated protein, 18kDa [Bos taurus]	1.15
281760	FABP5 fatty acid binding protein 5 [Bos taurus]	-1.51
616931	NUDT10 nudix (nucleoside diphosphate linked moiety X)-type motif 10 [Bos taurus]	-1.40
504287	SOAT1 sterol O-acyltransferase 1 [Bos taurus]	-1.82
281400	PHYH phytanoyl-CoA 2-hydroxylase [Bos taurus]	1.84
615390	ACAA2 acetyl-Coenzyme A acyltransferase 2 [Bos taurus]	3.34
281785	GLUD1 glutamate dehydrogenase 1 [Bos taurus]	-1.34
532512	POR cytochrome P450 reductase [Bos taurus]	-2.24
282318	PPP1CC protein phosphatase 1, catalytic subunit, gamma isoform [Bos taurus]	-1.61
618278	COMT catechol-O-methyltransferase [Bos taurus]	-2.27
614215	DDX39 DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 [Bos taurus]	-2.47
100138017	LOC100138017 similar to protein tyrosine phosphatase, receptor type, A [Bos taurus]	-2.77
614196	LACTB lactamase, beta [Bos taurus]	1.95
282199	COX6A1 cytochrome c oxidase subunit Via polypeptide 1 [Bos taurus]	-2.15
538060	LOC538060 similar to Aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) [Bos taurus]	-3.03
533020	CBR4 carbonyl reductase 4 [Bos taurus]	-2.54
540559	LANCL1 LanC lantibiotic sythetase component C-like 1 (bacterial) [Bos taurus]	1.49
281090	COX4 cytochrome c oxydase subunit 4 [Bos taurus]	3.69
Binding		
767990	ATXN10 ataxin 10 [Bos taurus]	-2.42
617531	LOC617531 similar to Calcium and integrin binding family member 2 [Bos taurus]	-1.93
407176	KCNMB1 potassium large conductance calcium-activated channel, subfamily M, beta member 1 [Bos taurus]	1.77
507319	EFNA1 ephrin-A1 [Bos taurus]	-1.78
520939	LOC520939 similar to Krueppel-like factor 2 (Lung krueppel-like factor) [Bos taurus]	-1.62
532571	WWC2 WW and C2 domain containing 2 [Bos taurus]	-2.40
538880	CPEB2 cytoplasmic polyadenylation element binding protein 2 [Bos taurus]	-2.14
613634	LSM4 LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae) [Bos taurus]	-1.76
614921	NUTF2 nuclear transport factor 2 [Bos taurus]	-1.62
507541	AMFR autocrine motility factor receptor [Bos taurus]	2.43
513331	PHF11 PHD finger protein 11 [Bos taurus]	-0.97
506534	CNPY2 canopy 2 homolog (zebrafish) [Bos taurus]	-2.95
786844	MXRA5 matrix-remodelling associated 5 [Bos taurus]	-1.45
526027	SPTBN1 spectrin, beta, non-erythrocytic 1 [Bos taurus]	-3.26
533233	APC adenomatous polyposis coli [Bos taurus]	-1.16
509108	RPS19BP1 ribosomal protein S19 binding protein 1 [Bos taurus]	-1.92
Transport		
506738	NUP155 nucleoporin 155kDa [Bos taurus]	-2.17
510872	NCBP2 nuclear cap binding protein subunit 2, 20kDa [Bos taurus]	-1.82
9444	QKI quaking homolog, KH domain RNA binding (mouse) [Homo sapiens]	-1.04
280813	HBB hemoglobin, beta [Bos taurus]	-1.53
534150	OPTN optineurin [Bos taurus]	-1.79
327673	NDUFA1 NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa [Bos taurus]	0.94
616979	RAB31 RAB31, member RAS oncogene family [Bos taurus]	-1.90
528100	SERP1 stress-associated endoplasmic reticulum protein 1 [Bos taurus]	-1.96
282177	CHRNA5 cholinergic receptor, nicotinic, alpha 5 [Bos taurus]	1.28

Multicellular organismal process		
768039	HOXA5 homeobox A5	2.28
Structural molecule activity		
615087	TUBB tubulin, beta [Bos taurus]	-1.08
282188	COL1A2 collagen, type I, alpha 2 [Bos taurus]	1.98
526516	HOOK2 hook homolog 2 (Drosophila) [Bos taurus]	-1.88
407121	NEB nebulin [Bos taurus]	-2.13
Immunological process		
613667	CXCL3 chemokine (C-X-C motif) ligand 3 [Bos taurus]	2.69
511603	PDIA5 protein disulfide isomerase family A, member 5 [Bos taurus]	-1.14
286793	PRDX2 peroxiredoxin 2 [Bos taurus]	-1.00
618411	LILRA4 leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4 [Bos taurus]	-2.07
511486	TRAα T cell receptor, alpha [Bos taurus]	-2.11
510798	TNFRSF10A tumor necrosis factor receptor superfamily, member 10a [Bos taurus]	1.72
652614	LOC652614 similar to HLA class I histocompatibility antigen, A-11 alpha chain precursor (MHC class I antigen A*11) [Bos taurus]	3.30
528862	DNAJA1 DnaJ (Hsp40) homolog, subfamily A, member 1 [Bos taurus]	-0.94
Unknown ontology		
533177	SCARB2 scavenger receptor class B, member 2 [Bos taurus]	-2.20
506530	SSR signal sequence receptor, beta (translocon-associated protein beta) [Bos taurus]	-2.47
505336	EPN2 epsin 2 [Bos taurus]	-2.19
511763	SYNCRIP synaptotagmin binding, cytoplasmic RNA interacting protein [Bos taurus]	-1.04
781257	MTCH1 mitochondrial carrier homolog 1 (C. elegans) [Bos taurus]	-1.00
510842	LPCAT4 lysophosphatidylcholine acyltransferase 4 [Bos taurus]	-2.43
509503	C19H17ORF39 chromosome 17 open reading frame 39 ortholog [Bos taurus]	-1.53
533820	MEGF9 similar to multiple EGF-like-domains 9 [Bos taurus]	-2.44
715086	LOC715086 similar to serine/threonine kinase 2 [Macaca mulatta]	-1.92
509776	WDR32 WD repeat domain 32 [Bos taurus]	-2.15
539480	TOLLIP toll interacting protein [Bos taurus]	-1.88
100153256	LOC100153256 similar to Serine/threonine-protein phosphatase 6 (PP6) [Sus scrofa]	1.35
512321	LOC512321 similar to missing oocyte CG7074-PA [Bos taurus]	-2.53
512327	DHX15 DEAH (Asp-Glu-Ala-his) box polypeptide 15 [Bos taurus]	-2.24
533595	TLE2 transducin-like enhancer of split 2 (E(sp1) homolog, Drosophilia) [Bos taurus]	-1.77
100138550	LOC100138550 similar to FIP1-like 1 [Bos taurus]	-2.38
616526	BET1 blocked early in transport 1 homolog (S. cerevisiae) [Bos taurus]	-1.26
619052	LOC619052 similar to hCG2041454 [Bos taurus]	-2.35
519736	RANBP10 RAN binding protein 10 [Bos taurus]	-1.59
513309	GFM1 G elongation factor, mitochondrial 1 [Bos taurus]	-1.38
785762	LOC785762 similar to prostaglandin F synthase-like 1 protein [Bos taurus]	-3.05
475318	MFSD2 major facilitator superfamily domain containing 2 [Canis lupus familiaris]	-2.03
519172	BLOC1S2 biogenesis of lysosomal organelles complex-1, subunit 2 [Bos taurus]	-1.58
787566	LOC787566 similar to High mobility group protein B1 (High mobility group protein 1) (HMG-1) [Bos taurus]	-1.87
533520	OCIAD1 OCIA domain containing 1 [Bos taurus]	-1.30
540910	SMARCE1 SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1 [Bos taurus]	-1.34
512094	ALKBH4 alkB, alkylation repair homolog 4 (E. coli) [Bos taurus]	2.52
100126445	DGCR6L DiGeorge syndrome critical region gene 6-like [Bos taurus]	-2.46
282658	ARPP-19 cAMP-regulated phosphoprotein, 19 kD[Bos taurus]	-1.53
100125763	LOC100125763 neuronal protein 3.1 [Bos taurus]	-1.38
508569	LOC508569 similar to CG5913 CG5913-PA [Bos taurus]	2.55
508149	CCDC58 coiled-coil domain containing 58 [Bos taurus]	-1.02
505238	RALB v-ral simian leukemia viral oncogene homolog B (ras relatated; GTP binding protein) [Bos taurus]	-2.75

510412	GOLGA5 golgi autoantigen, golgin subfamily a, 5 [Bos taurus]	-1.20
505320	ARIH2 ariadne homolog 2 (Drosophila) [Bos taurus]	-1.62
506622	TMEM219 transmembrane protein 219 [Bos taurus]	-1.80
617968	C25H7ORF47 chromosome 7 open reading frame 47 ortholog [Bos taurus]	-1.92
613425	UROS uroporphyrinogen III synthase [Bos taurus]	-1.18
100153616	LOC100153616 similar to WHSC1L1 protein [Sus scrofa]	-2.27
dB EST ID	**Clone Name**	**M value**
2834671	1367982 MARC 7BOV Bos taurus cDNA 5', mRNA sequence	2.90
17191506	695769 MARC 6BOV Bos taurus cDNA 5', mRNA sequence	-2.24
35786314	000829BEMN005742HT BEMN Bos taurus cDNA 5', mRNA sequence	-1.71
17179146	608349 MARC 6BOV Bos taurus cDNA 3', mRNA sequence	-2.13
41875419	A95041A FFB Bos taurus cDNA clone A9504 3', mRNA sequence	3.01
48105736	LB03351.C21.1_L07 GC_BGC-33 Bos taurus cDNA clone IMAGE:8722521	-2.26
23533146	UMC-bend_0B02-018-e07 Day 16 Uterus from a non-pregnant animal bend Bos taurus cDNA 3', mRNA sequence	-2.20
24791078	UMC-bcl_0A01-006-f02 Corpora Lutea (CL) Bos taurus cDNA 3', mRNA sequence	-1.44
24792343	UMC-bend_0B01-025-g11 Day 16 Uterus from a pregnant animal bend Bos taurus cDNA 3', mRNA sequence	-1.59
48110166	LB03418.C21_O13 GC_BGC-34 Bos taurus cDNA clone IMAGE:8660391 3', mRN sequence	-1.44
24792640	UMC-bend_0B02-024-g04 Day 16 Uterus from a pregnant animal bend Bos taurus cDNA 3', mRNA sequence	-2.42
43569254	LB01840.CR_P11 GC_BGC-18 Bos taurus cDNA clone IMAGE:8830909 5', mRNA sequence	-2.10
26586667	UMC-bcl_0A02-020-g02 Day 14 CL from a pregnant animal bcl Bos taurus cDNA 3', mRNA sequence	-0.93
26586705	UMC-bcl_0A02-022-c06 14 CL from a pregnant animal bcl Bos taurus cDNA 3',mRNA sequence	-1.13
26587424	UMC-bcl_0B01-019-e10 14 CL from a non-pregnant animal bcl Bos taurus cDNA 3', mRNA sequence	-2.31
53808734	C0009504K17.Q1KM13R KN510 Bos taurus rectum from E. coli unchallenged animals Bos taurus cDNA clone C0009504K17 3', mRNA sequence	-2.28
26591099	UMC-bemiv_0B01-006-e09 Early Embryo In Vitro Produced bemiv Bos taurus cDNA 3', mRNA sequence	-1.94
41933278	L97921A FNM Bos taurus cDNA L9792 3', mRNA sequence	-1.88
26591916	UMC-bemiv_0B02-008-h03 Pre-compaction morula in vitro produced bemiv Bos taurus cDNA 3', mRNA sequence	-1.85
48104002	LB03326.C21.1_N17 GC_BGC-33 Bos taurus cDNA clone IMAGE:8712979 3',mRNA sequence	-2.00
26594691	UMC-bmix_0B02-004-d10 Day 16 Conceptus bmix Bos taurus cDNA 3', mRNA sequence	2.71
26594691	UMC-bof_0A02-003-h02 Ovarian follicle recruited bof Bos taurus cDNA 3', mRNA sequence	-2.59
26595022	UMC-bof_0B02-001-h08 Ovarian follicle pre-ovulatory bof Bos taurus cDNA 3',mRNA sequence	1.72
36369084	1602252 MARC 11BOV Bos taurus cDNA 3', mRNA sequence	-1.88
27655206	UMC-bcl_0A01-024-c08 Day 8 Corpus luteum bcl Bos taurus cDNA 3', mRNA sequence	-1.37
27655210	UMC-bcl_0A01-024-d02 Day 8 Corpus luteum bcl Bos taurus cDNA 3', mRNA sequence	-1.33
48108485	LB03411.c21_D17 GC_BGC-34 Bos taurus cDNA clone IMAGE:8657443 3', mRNA sequence	1.82
35858054	010128BEMN061945HT BEMN Bos taurus cDNA 5', mRNA sequence	1.33
27662271	UMC-bof_0A02-008-a10 Ovarian follicle pre-ovulatory bof Day 8 Corpus luteum bcl Bos taurus cDNA 3', mRNA sequence	-2.31
48106176	LB03354.C21_P09 GC_BGC-33 Bos taurus cDNA clone IMAGE: 8723771 3', mRNA sequence	0.92
28484716	UMC-bend_0B01-028-b04 Day 16 Uterus from a pregnant animal bend Bos taurus cDNA 3' mRNA sequence	-0.90
43575584	LB02731.CR.2_P19 GC_BGC-27 Bos taurus cDNA clone IMAGE:8621637 5', mRNA sequence	-1.36
28486526	UMC-bof-0B01-013-b07 Ovarian follicle recruited bof Bos taurus cDNA 3', mRNA sequence	2.80
28666712	UMC-bemiv_0B01-029-b09 In virto derived blastocytes Day 8 bemiv Bos taurus cDNA 3', mRNA sequence	-1.18
28665778	UMC-bemic_0A01-027-c09 Metaphase II stage oocyte bemiv Bos taurus cDNA 3', mRNA sequence	-4.21

Table 4: Differentially expressed genes in SCF grouped based on ontology – molecular function and biological process

Entrez ID	Gene Name	M value
Proteolysis/protein modification/catabolism		
512484	TYK2 tyrosine kinase 2 [Bos taurus]	-1.37
54954	FAM120C family with sequence similarity 120C [Homo sapiens]	0.96
614817	PSMG1 proteasome (prosome, macropain) assembly chaperone 1 [Bos taurus]	-0.99
415116	PIM3 pim-3 oncogene [Homo sapiens]	-1.63
538284	USP15 ubiquitin specific peptidase 15 [Bos taurus]	0.93
533874	PSMB3 proteasome (prosom, macropain) subunit, beta type, 3 [Bos taurus]	-1.63
510796	LONP1 Ion peptidase 1, mitochondrial [Bos taurus]	-0.91
528919	FBXW12 F-box & WD repeat domain containing 12 [Bos taurus]	1.04
280692	HP haptoglobin [Bos taurus]	-0.97
Apoptosis		
444864	PEG3 paternally expressed 3 [Bos taurus]	1.89
Cellular process		
524821	SFT2DA SFT2 domain containing 1 [Bos taurus]	1.32
790411	LOC790411 endonuclease reverse transcriptase [Bos taurus]	1.04
281790	GNAI1 guanine nucleotide binding protein (G protein), α inhibiting activity polypeptide 1 [Bos taurus]	1.03
509717	CCNC cyclin C [Bos taurus]	-1.66
514407	GMFG glia maturation factor, gamma [Bos taurus]	-3.15
282637	CDH1 cadherin 1, type 1, E-cadherin (epithelial) [Bos taurus]	2.08
443076	C-MET growth factor receptor c-met [Ovis aries]	1.60
Translation		
23312	DMXL2 Dmx-like 2 [Homo sapiens]	0.98
286884	RPS29 ribosomal protein S29 [Bos taurus]	-0.90
508948	RSL1D1 ribosomal L1 domain containing 1 [Bos taurus]	-1.44
614579	MRPL14 mitochondrial ribosomal protein L14 [Bos taurus]	-1.01
Trascription		
7003	TEAD1 TEA domain family member 1 (SV40 transcriptional enhancer factor)[Homo sapiens]	2.11
55274	PHF10 PHD finger protein 10 [Homo sapiens]	1.16
535026	CHD2 chromodomain helicase DNA binding protein 2 [Bos taurus]	-1.38
Metabolism		
281103	CTGF connective tissue growth factor [Bos taurus]	1.67
281748	ECHS1 enoyl Coenzyme A hydratase, short chain, 1, mitochondrial [Bos taurus]	0.96
507988	CYP3A4 cytochrome P450, subfamily IIIA, polypeptide 4 [Bos taurus]	-1.27
512112	AGPAT2 1-acylglycerol-3-phosphate-O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) [Bos taurus]	-0.90
327696	SDHC succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa [Bos taurus]	1.26
505049	AMY2A amylase, alpha 2A (pancreatic) [Bos taurus]	1.26
Binding		
506275	WBP4 WW domain binding protein 4 (formin binding protein 21) [Bos taurus]	2.36
513047	ING1 inhibitor of growth family, member 1 [Bos taurus]	-0.92
617552	CNKSR1 connector enhancer of kinase suppressor of Ras 1 [Bos taurus]	-1.21
282196	CORO1A coronin, actin binding protein, 1A [Bos taurus]	-1.89
534583	CNN1 calponin 1, basic, smooth muscle [Bos taurus]	1.84
538742	MIER1 mesoderm induction early response 1 homolog (Xenopus laevis) [Bos taurus]	1.34
536944	CLEC1A C-type lectin domain family 1, member A [Bos taurus]	1.24
532635	RCOR3 REST corepressor 3 [Bos taurus]	1.83
327666	SIRPA signal-regulatory protein alpha [Bos taurus]	-1.20
100139498	LOC100139498 similar to DNA replication licensing factor MCM9 (Mini-chromosome maintenance deficient 9) (hMCM9) (Mini-chromosome maintenance deficient domain containing protein 1) [Bos taurus]	-1.92
613381	HN1 hematological and neurological expressed 1 [Bos taurus]	-1.04
509108	RPS19BP1 ribosomal protein S19 binding protein 1 [Bos taurus]	-0.92

Transport		
8411	EEA1 early endosome antigen 1 [Homo sapiens]	1.54
529929	DDX19A DEAD (Asp-Glu-Ala-As) box polypeptide 19A [Bos taurus]	0.95
282187	COL1A1 collagen, type I, alpha 1 [Bos taurus]	1.38
282523	SLC25A11 solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11 [Bos taurus]	-1.15
286861	FTL ferritin, light polypeptide [Bos taurus]	-0.90
511800	ANKH ankylosis, progressive homolog (mouse) [Bos taurus]	1.04
100125922	RER1 RER1 retention in endoplasmic reticulum 1 homolog (S. cerevisiae) [Bos taurus]	-3.30
26207	PITPNC1 phosphatidylinositol transfer protein, cytoplasmic 1 [Homo sapiens]	-1.19
Multicellular organismal processes		
3696	ITGB8 integrin, beta 8 [Homo sapiens]	1.76
22858	ICK intestinal cell (MAK-like) kinase [Homo sapiens]	2.54
768039	HOXA5 homeobox A5 [Bos taurus]	1.20
Immunological response		
282167	CATHL5 cathelicidin 5 [Bos taurus]	-2.06
539220	AHSA1 AHA1, activator of heat shock 90kDa protein ATPase homolog1 (yeast) [Bos taurus]	-1.19
615833	IFITM2 interferon induced transmembrane protein 2 (1-8D) [Bos taurus]	-0.99
616015	C11H9orf16 chromosome 9 open reading frame 16 ortholog [Bos taurus]	-1.14
767928	RBPJ recombination signal binding protein for immunoglobulin kappa J region [Bos taurus]	-1.85
539674	TNFAIP1 tumor necrosis factor, alpha-induced protein 1 (endothelial) [Bos taurus]	1.94
613449	DCLK1 doublecortin-like kinase 1 [Bos taurus]	1.30
618530	C13H20ORF52 chromosome 20 open reading frame 52 ortholog [Bos taurus]	-0.91
540085	GAB1 GRB2-associated binding protein 1 [Bos taurus]	1.03
529115	HIVEP1 human immunodeficiency virus type 1 enhancer binding protein 1 [Bos taurus	1.91
613288	WNT11 wingless-type MMTV integration site family, member 11 [Bos taurus]	-1.08
	ABL61276.1TCGR5 T cell receptor gamma c5 [Bos taurus]	1.04
Unknown ontology		
100140540	LOC100140540 hypothetical protein LOC100140540 [Bos taurus]	-0.91
782535	LOC782535 similar to Dmx-like 2 [Bos taurus]	0.98
767958	C29H11orf10 chromosome 11 open reading frame 10 ortholog [Bos taurus]	-0.91
505843	LOC50583 hypothetical protein LOC505843 [Bos taurus]	-1.81
100157539	LOC100157539 similar to SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2 [Sus scrofa]	-1.92
338325	XIST X (inactive)-specific transcript [Bos taurus]	1.90
522372	SYF2 SYF2 homolog, RNA splicing factor (S. cerevisiae) [Bos taurus]	-1.32
768057	HIGD1A HIG1 domain family, member 1A [Bos taurus]	-0.90
501569	RGD1564799 similar to transmembrane 4 superfamily member 10 [Rattus norvegicus]	1.35
781772	LOC781772 similar to RIKEN cDNA 2200001I15 [Bos taurus]	1.28
788792	FARSB phenylalanyl-tRNA synthetase, beta subunit [Bos taurus]	-0.97
508905	ZRANB2 zinc finger, RAN-binding domain containing 2 [Bos taurus]	1.00
616069	N4BP2L1 NEDD4 binding protein 2-like 1 [Bos taurus]	1.96
534400	CCDC100 coiled-coil domain containing 100 [Bos taurus]	1.62
525698	C8H4orf27 chromosome 4 open reading frame 27 ortholog [Bos taurus]	1.17
10007274	ARID5B AT rich interactive domain 5B (MRF1-like) [Equus caballus]	1.46
451412	LOC451412 similar to KIAA0280 [Pan troglodytes]	1.13
506521	MRPL41 mitochondrial ribosomal protein L41 [Bos taurus]	-1.11
522515	SGPL1 sphingosine-1-phosphate lyase 1 [Bos taurus]	0.91
541187	ATP8B1 ATPase, class I, type 8B, member 1 [Bos taurus]	1.12
507010	COL16A1 collagen, type XVI, alpha 1 [Bos taurus]	1.01
dB EST ID	**Clone Name**	**M value**
21994317	4110245 BARC 9BOV Bos taurus cDNA clone 9BOV38_017 5', mRNA sequence	1.14
21609756	UMC-bov-0A01-008-d07 Day 3 Oviduct bov Box taurus cDNA 3', mRNA sequence	1.07
28364486	1367783 MARC 7BOV Bos taurus cDNA 5', mRNA sequence	1.85
21608729	UMC-bend_0B01-007-a02 Day 8 Uterus bend Bos taurus cDNA 3', mRNA sequence	1.38

21758041	UMC-bov_0A02-009-e07 Day 3 Oviduct bov Bos taurus cDNA 3', mRNA sequence	2.14
33993319	Hw_Fat_9_B09 Bos taurus CF-24-HW fat cDNA library Bos taurus cDNA, mRNA sequence	0.94
48013890	LB02354.CR_H03 GC_BGC-23 Bos taurus cDNA clone IMAGE:8610293 5', mRNA sequence	1.00
41881472	B28691A FFB Bos taurus cDNA clone B2869 3', mRNA sequence	1.52
53805706	C0009290H03.Q1KM13R KN510 Bos taurus cDNA clone C0009290H03 3', mRNA sequence	1.85
48119692	LB0343.C21_L04 GC_BGC-34 Bos taurus cDNA clone IMAGE:9109590 3', mRNA sequence	0.90
48064420	LB03545.CR_F24 GC_BGC-35 Bos taurus cDNA clone IMAGE:8670554 5', mRNA sequence	1.73
22983063	UMC-bend_0B02-012-b06 Uterus (endometrium) bend Bos taurus cDNA 3', mRNA sequence	-1.46
43741903	LB004118.CR_P03 GC_BGC-04 Bos taurus cDNA clone IMAGE:8975669 5', mRNA sequence	1.77
53804499	C0009288L06.Q1KM13R KN510 M. bovis infected Bos taurus DC/Mphage/Mo Bos taurus cDNA clone C0009288L06 3', mRNA sequence	-1.25
40867216	LB02329.CR.1_D21 GC_BGC-23 Bos taurus cDNA clone IMAGE:8270567, mRNA sequence	2.58
48010098	LB02340.CR_M09 GC_BGC-23 Bos taurus cDNA clone IMAGE:8605043 5', mRNA sequence	1.02
41965501	P98231A FNM Bos taurus cDNA clone P9823 3', mRNA sequence	0.92
48152781	LB02353.C21_M01 GC_BGC-23 Bos taurus cDNA clone IMAGE:8610027 3', mRNA sequence	1.32
43539218	LB01637.CR.1_H13 GC_BGC-16 Bos taurus cDNA clone IMAGE:8325279 5', mRNA sequence	1.61
24792838	UMC-bend_0B02-027-d05 Day 16 Uterus from a pregnant animal bend Bos taurus cDNA 3', mRNA sequence	-1.39
43569254	LB01840.CR_P11 GC_BGC-18 Bos taurus cDNA clone IMAGE:8830909 5', mRNA sequence	1.25
48116062	LB03449.C21_C09 GC_BGC-34 Bos taurus cDNA clone IMAGE:8652035 3', mRNA sequence	1.13
36367604	1598932 MARC 11BOV Bos taurus cDNA 3', mRNA sequence	1.75
43544222	LB01652.CR_I18 GC_BGC-16 Bos taurus cDNA clone IMAGE:8385308 5', mRNA sequence	1.17
48108485	LB03411.C21_D17 GC_BGC-34 Bos taurus cDNA clone IMAGE:8657443 3', mRNA sequence	1.12
41890507	A37381A FFB Bos taurus cDNA clone A3738 3', mRNA sequence	0.96
53799211	C0009281B14.Q1KM13R KN510 Bos taurus uninfected DC/Mphage/Mo Bos taurus cDNA clone C0009281B14 3', mRNA sequence	1.00
53811547	C0009508L13.Q1KM13R KN510 Bos taurus rectum from E. coli challenged/treated animals Bos taurus cDNA clone C0009508L13 3', mRNA sequence	-0.96
53811547	C0009508L13.Q1KM13R KN510 Bos taurus rectum from E. coli challenged/treated animals Bos taurus cDNA clone C0009508L13 3', mRNA sequence	2.02
28667858	UMC-bof_0B02-018-h08 Ovarian follicle early selected bof Bos taurus cDNA 3',mRNA sequence	0.91
53809512	C0009505N10.P1KM13F KN510 Bos taurus rectum from E. coli unchallenged animals Bos taurus cDNA clone C0009505N10 5', mRNA sequence	1.06
41885977	B54791A FFB Bos taurus cDNA clone B5479 3', mRNA sequence	2.54
43745613	LB011127.CR_O21 GC_BGC-11 Bos taurus cDNA clone IMAGE:8971439 5', mRNA sequence	1.15

Figure 1: Comparison of directional changes of genes within the microarray used for validation. Microarray fold change (dark bar) and the fold change from the RT-PCR (light bar). Genes that end with a –M are from LM tissue and genes that end with a –F are from SCF tissue.

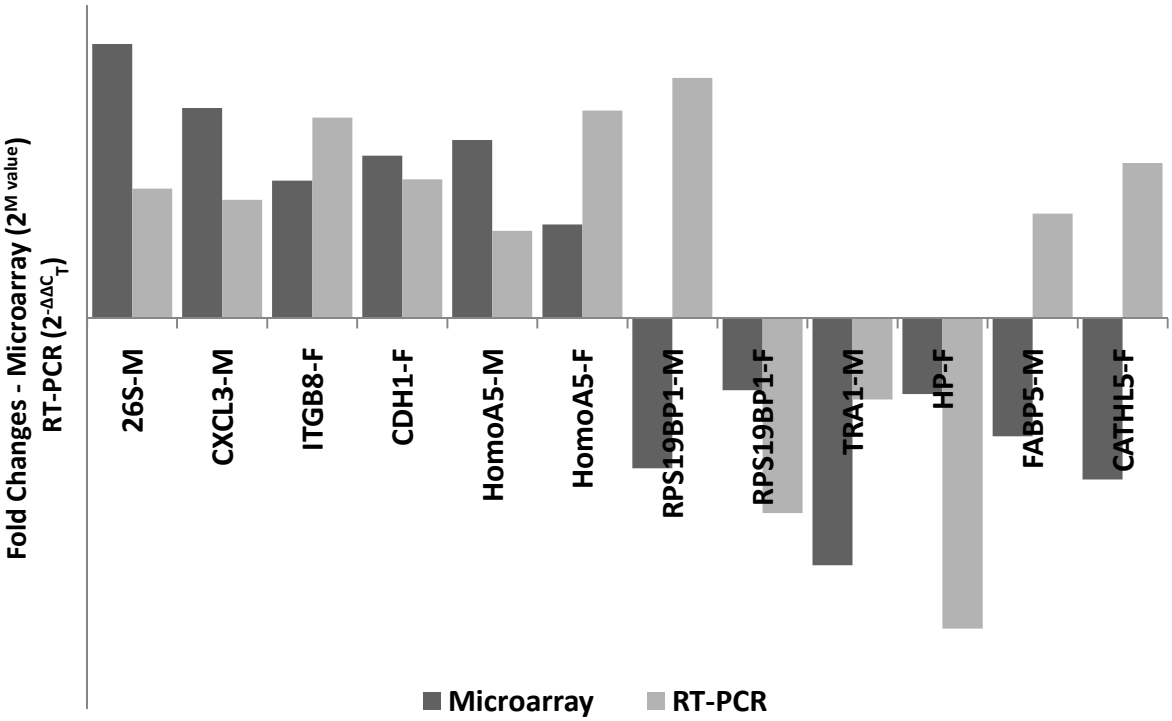


Figure 2: 26S proteasome gene expression in longissimus muscle of healthy (HLTH) and chronic (CHR) heifers.

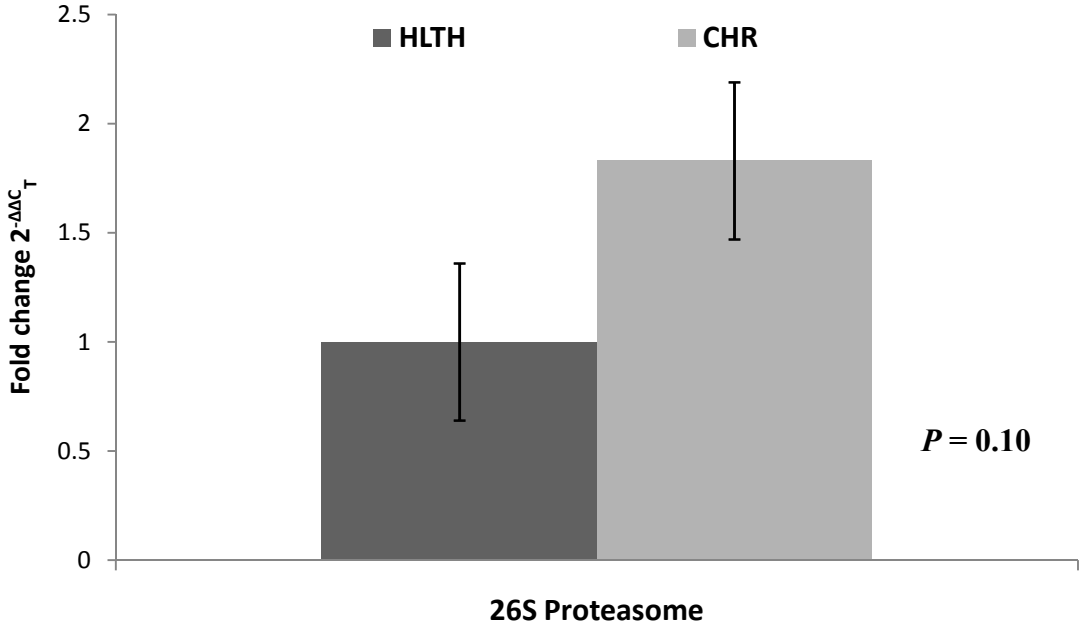


Figure 3: Haptoglobin gene expression in subcutaneous fat of healthy (HLTH) and chronic (CHR) heifers.

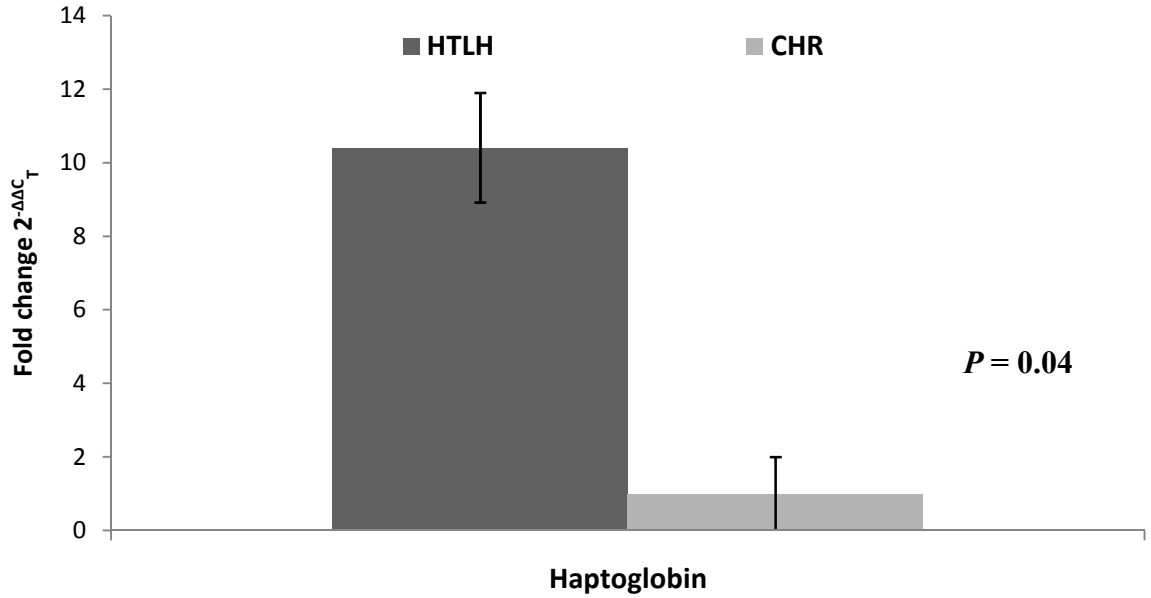


Figure 4: Ingenuity® Pathway Analysis of both tissues showing genes in the protein ubiquitin pathway, glucocorticoid receptor signaling, hepatic fibrosis/hepatic stellate cell activation, and PPARα activation. CP = Pathway identification connecting genes involved in specified pathway. Green colored shapes are down-regulated and red are up-regulated in the microarray. Solid lines show direct interactions. Dotted lines are indirect interactions.

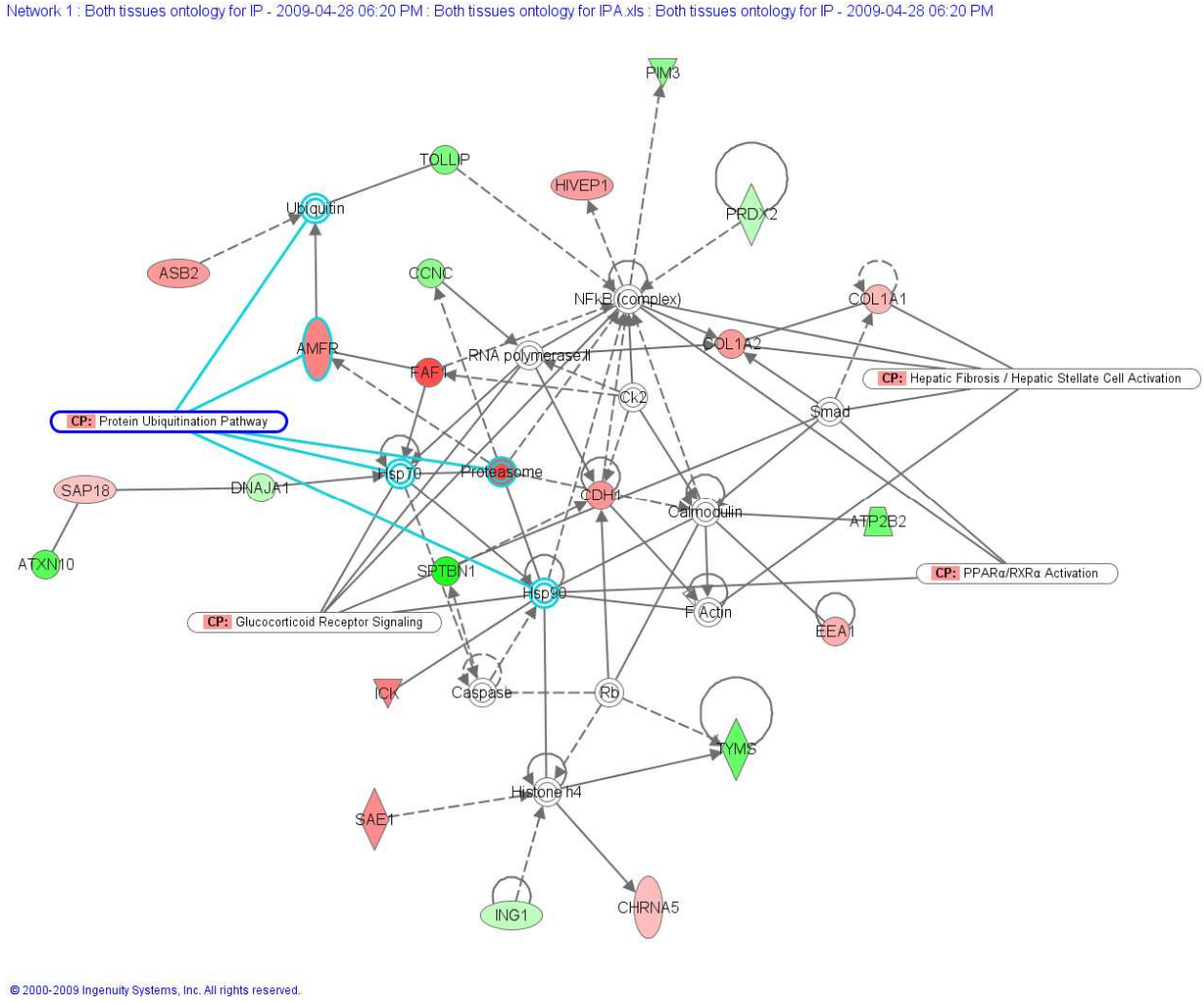


Figure 5: Ingenuity® Pathway Analysis from LM and SCF showing the central hub for hepatocyte nuclear factor 4, alpha (HNF4A). Solid lines show direct interactions. Dotted lines show indirect interactions. Green shapes are down-regulated genes from the microarray. Red shapes are up-regulated genes from the microarray.

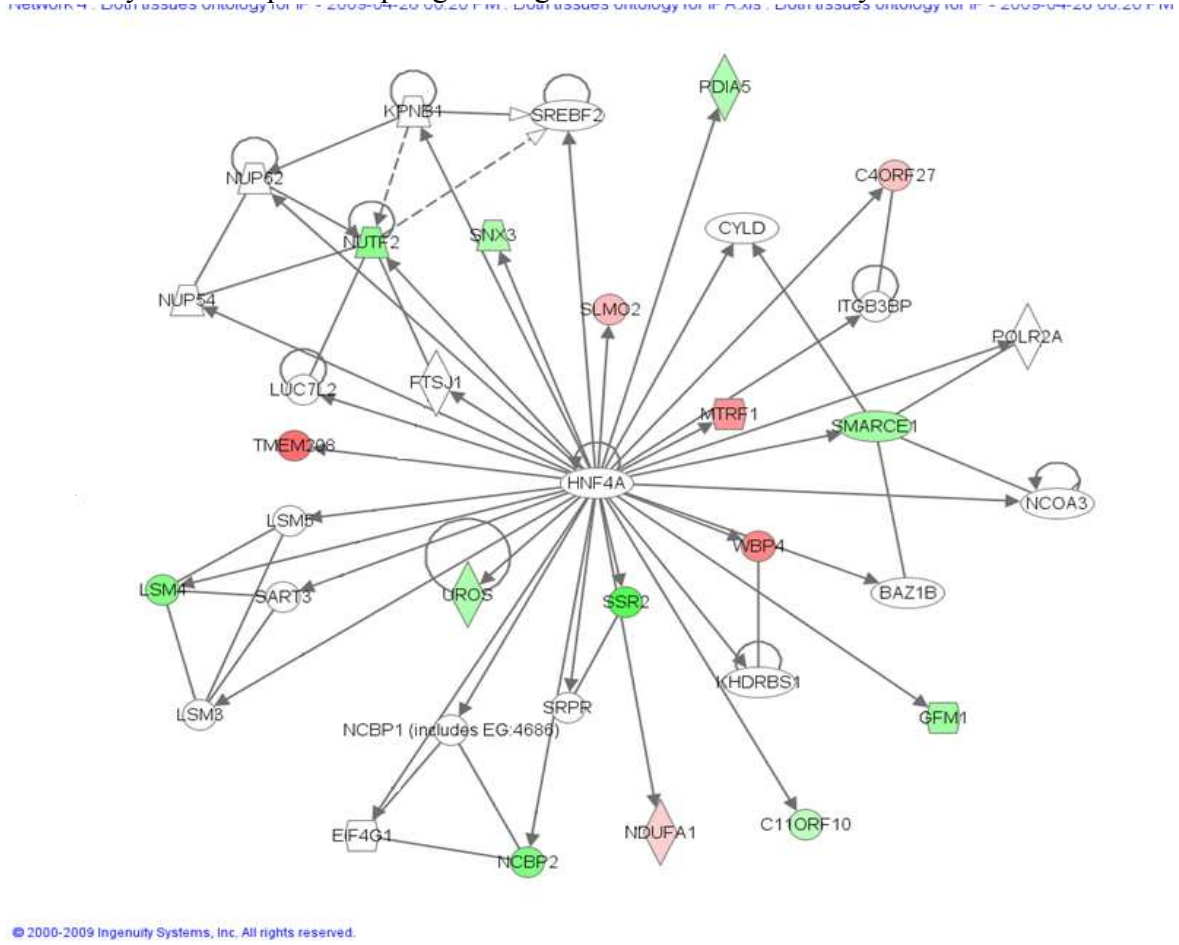
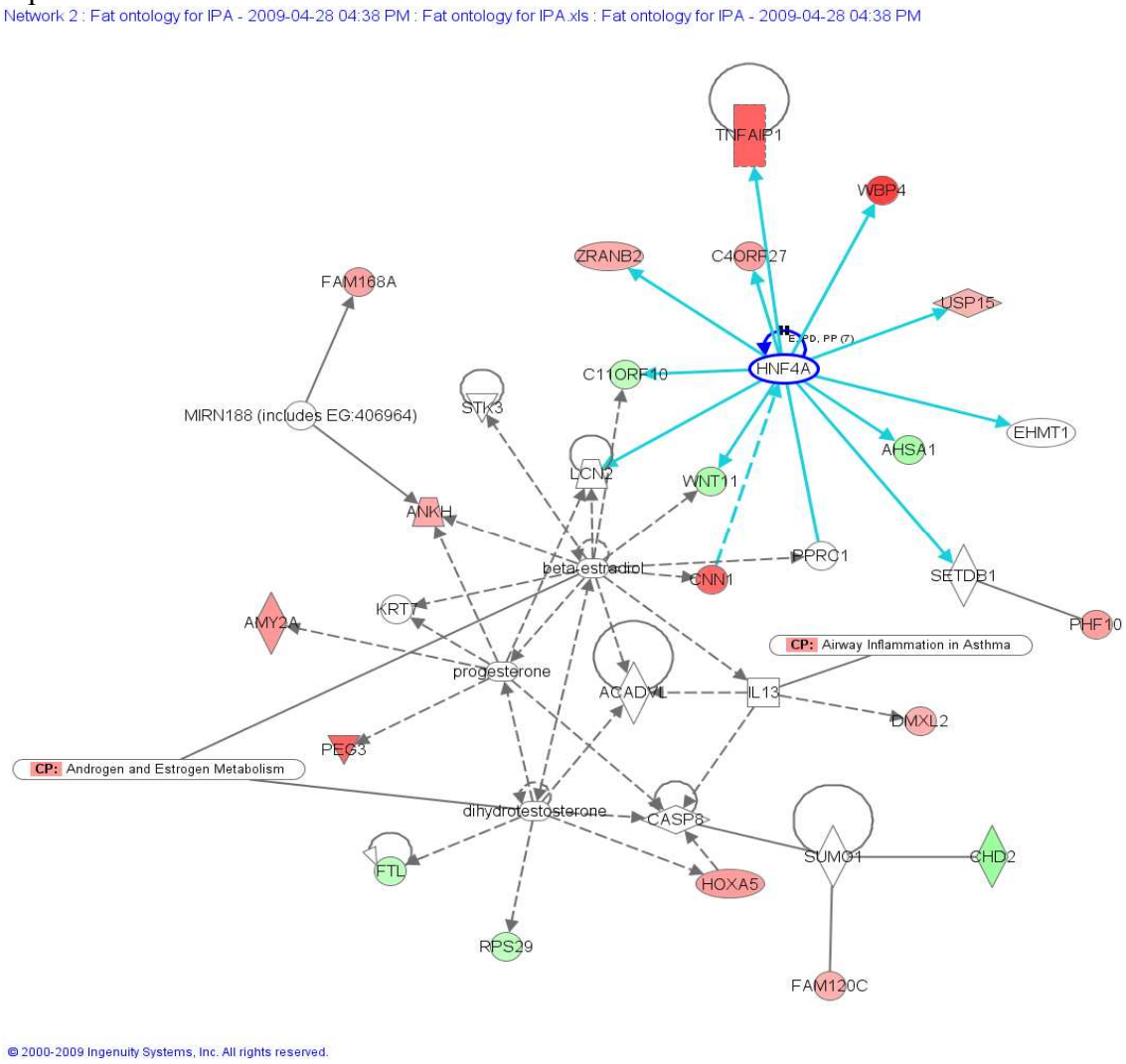


Figure 6: Ingenuity® Pathway Analysis of SCF showing a central hub for β-estradiol and hepatocyte nuclear factor 4, alpha (HNF4A) activation within the SCF tissue. Red colored shapes are up-regulated and green are down-regulated genes from this experiment. Solid lines show direct interactions. Dotted lines show indirect interactions.



CHAPTER IV

EFFECT OF NUMBER OF TIMES TREATED FOR CLINICAL SIGNS OF BOVINE RESPIRATORY DISEASE ON GENE EXPRESSION IN HEIFERS DURING A 63 DAY PRECONDITIONING PERIOD

J. Johnson*, D. R. Stein*, L. O. Burciaga-Robles*, B. P. Holland*, D. L. Step†, U. E. DeSilva*, J. Ritchey†, and C. R. Krehbiel*,[1]

*Department of Animal Science, Oklahoma State Univesity, Stillwater 74078 and †College of Veterinary Medicine, Oklahoma State University, Stillwater 74078

[1]Corresponding author: Dept. of Anim. Sci., 208 Animal Sci. Bldg., Stillwater, OK 74078; phone: 405/744-8856; fax: 405/744-7390; E-mail:clint.krehbiel@okstate.edu

ABSTRACT: Bovine respiratory disease (BRD) is the most costly disease to the cattle industry. The objective of this experiment was to determine the effects of number of antimicrobial treatments for BRD on gene expression in economically important tissues. Tissue biopsy samples from the longissimus dorsi muscle (LM) and subcutaneous fat (SCF) between the 12th and 13th rib from heifers never treated against BRD (HLTH; n = 5), treated once (T1; n = 5), treated twice (T2; n = 5), treated three times (T3; n = 5), and heifers classified as chronically morbid (CHR; n = 5) were collected after a 63-d preconditioning period. Chronic was defined as animals which had received three

antimicrobial treatments and had lost BW during the previous 21 d on feed. Genes (n = 14) were evaluated using quantitative RT-PCR. Proc Mixed procedures of SAS were used to evaluate significance of number of treatments. In SCF, components of the ubiquitin pathway, F-box and WD repeating domain containing 12 (FBXW12), tended ($P \leq 0.10$) to increase as the number of antimicrobial treatments increased. In addition, expression of FBXW12 tended ($P = 0.10$) to be greater and expression of 26S proteasome subunit ATPase 1 (26S) tended ($P = 0.09$) to be greater in LM of CHR heifers than HTLH heifers. In LM, fold change expression of tumor necrosis factor-α was greater ($P = 0.01$) in CHR heifers than HLTH, T1 and T2; T3 heifers were intermediate. Haptoglobin (HP) expression in SCF was greater ($P = 0.05$) in T1 than CHR heifers; HLTH, T2, and T3 heifers were intermediate. Results suggest that genes involved in muscle wasting via the ubiquitin pathways and expression of TNF-α may be activated in morbid heifers. These results could partially explain the decreased growth rates and carcass quality in cattle infected with BRD.

Key words: antimicrobial treatment, bovine respiratory disease, cattle, chronic, gene expression

INTRODUCTION

Bovine respiratory disease (**BRD**) is the most costly disease in feedlot cattle in the United States because it decreases performance and increases medical costs and potential carcass discounts. Clinical signs observed when an animal is experiencing BRD include, but are not limited to, anorexia, depression, and fever (Broussard et al., 2001). With

anorexia the nutritionally deprived animals lose BW resulting in decreased performance. Lehnert et al. (2006) used cattle nutritionally deprived for 114 days and observed BW loss-mediated muscle atrophy and manipulation of fatty acid profiles. Therefore, Lehnert et al. (2006) proposed that low feed intake, such as in calves experiencing BRD, could partially explain long-term decreases in performance and carcass merit.

Muscle cells have been shown to express receptors for both cytokines and growth factors (Broussard et al., 2003). The magnitude of increases in inflammatory cytokines during acute respiratory infection may lead to decreases in protein accretion and growth (Escobar et al., 2004). Cytokines have also been shown to act on skeletal muscle to decrease the efficacy of anabolic hormones such as insulin-like growth factor I (**IGF-I**), while also directly influencing the synthesis and degradation of skeletal muscle protein (Broussard et al., 2001; Alvarez et al., 2002; Escobar et al., 2004). Skeletal muscle may be an important source of both catabolic and anti-inflammatory cytokines and the balance between these two could possibly be involved in the modulation of protein turnover and muscle wasting (Alvarez et al., 2002). Muscle tissue is not only used in contractile functions, but also as a protein reservoir that can be mobilized in a stressed state (Cao et al., 2005).

Recent evidence indicates that adipocytes and myofibers are equipped with functional pattern recognition receptors, and are capable of responding directly to pathogens and other receptor ligands. Adipocytes and myofibers, acting as functional pattern recognition receptors, are active participants in the innate immune response producing a number of immune and metabolic regulators, including pro-inflammatory cytokines and adiponectin which help in regulating homeostasis (Mohamed-Ali et al.,

1998; Havel et al., 2002; Ding et al., 2004; Gabler and Spurlock, 2007). For example, in adipocytes and muscle cells nuclear factor-kappa B (**NF-κB**) is activated by bacterial lipopolysaccharide and saturated fatty acids that are recognized by toll-like receptor-4 (**TLR-4**) pattern recognition which results in local production of tumor necrosis factor-α (**TNF-α**) and interleukin 6 (**IL-6**). Adipocytes also work in regulation by being the source of adiponectin, an anti-inflammatory hormone, which suppresses the activation of NF-κB (Gabler and Spurlock, 2007). Toll-like receptor-4 is part of a large family of receptors that recognize pathogen-associated molecular patterns (Frost et al., 2002). Toll-like receptors are sensors of microbial infection and are responsible for the induction of both innate and adaptive immune responses. The mammalian TLR family presently consists of ten members, and because of all the many receptors, it provides the immune system with the ability to respond to a wide variety of pathogens. Toll-like receptor-4 signaling can stimulate IL-6 mRNA expression in mouse skeletal muscle. Frost et al. (2003) observed that mice that have a mutation in the TLR-4 receptor have a greatly decreased expression of IL-6.

Nuclear factor-kappa B is the best defined transcription factor that is activated by TLR (Toubi and Shoenfeld, 2004). Nuclear factor-kappa B is most commonly known to be involved in innate and adaptive immunity (Langland et al., 2006; de los Santos et al., 2007; Takeuchi and Akira, 2007). Nuclear factor-kappa B has been shown to induce the expression of IL-1β, TNF-α, and IL-8 in bovine alveolar macrophages exposed to *M. haemolytica* leukotoxin and endotoxin (Hsuan et al., 1999; Caverly et al., 2003). Therefore, NF-κB is a central integration site for pro-inflammatory signals and a regulator of related target genes which include cytokines and chemokines, immune-

presenting receptors, antigen-presenting receptors, stress response and acute phase proteins, and other regulators of apoptosis, growth, and host defense (Cai et al., 2004).

Tumor necrosis factor-α is known to be important in mediating inflammation and cytotoxic reactions and currently it has been thought to have an active role in metabolism. Broussard et al. (2003) observed that when IGF-I receptors were stimulated by concentrations of lower than 0.1 ng/mL of TNF-α, it inhibited *de novo* protein synthesis by 50% in myoblasts. Tumor necrosis factor-α has been shown, in chronically high concentrations, to result in muscle wasting in skeletal muscle due to increased protein turnover (Llovera et al., 1993).

Cystic fibrosis (**CF**) is a disease in humans that is caused by a genetic defect that impairs the mucous lining of the lungs. This predisposes patients to chronic bacterial infection in the respiratory tract that causes lung destruction and loss of pulmonary function. Studies suggest that the host inflammatory and metabolic responses to chronic pulmonary infection have an impact on maintenance of body composition (Pencharz et al., 1984; Stutts et al., 1986; Elborn et al., 1993; Bell et al., 1996; Bell et al., 2000; Ionescu et al., 2000; Ionescu et al., 2002). Changes in body composition are noted to possibly come from a negative energy balance resulting from an inadequate energy intake to meet energy demands. Ionescu et al. (2002) summarized that excessive energy expenditure in CF may result from the increased energy cost of breathing due to altered pulmonary mechanics, catabolic intermediary metabolism due to chronic pulmonary infection, or an acute phase inflammatory response with increased circulating immunoreactive TNF-α, IL-1, IL-6, and other counter-regulatory hormones. Several studies have shown that cytokines regulate the catabolic response with mobilization of fat

and skeletal muscle used as alternative energy sources (Van der Poll and Sauerwein, 1993; Ionescu et al., 2002).

An experiment conducted by Montgomery et al. (2009) showed that during a 36-d receiving period ADG decreased linearly with increases in the number of times an animal was treated for clinical signs of BRD. This resulted in heifers with lighter BW going into the grazing period, the subsequent finishing phase, and at slaughter. Carcasses were lighter, while fat thickness and marbling scores were lower with increases in the number of times treated. There was also a tendency for decreased LM area (Montgomery et al., 2009).

Because BRD infections lead to decreased performance and carcass merit of cattle, our objective was to determine if the number of antimicrobial treatments for clinical signs of BRD affects gene expression in muscle and fat tissues.

METHODS AND MATERIALS

The experimental protocol was approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Animals

Three hundred sixty high-risk heifers (initial BW = 248 ± 14 kg) were purchased from multiple auction barns in Kentucky and delivered to the Willard Sparks Beef Research Feedlot (**WSBRF**), Stillwater, OK. The heifers were individually identified and ear-notched to test for persistent infection with bovine viral diarrhea virus as described by Fulton et al. (2006). A heifer identified as PI was removed from the group after arrival at the WSBRF. Upon arrival, heifers were weighed and given *ad libitum*

access to prairie hay. Within 24 hours of arrival heifers were processed. Initial processing consisted of administration of a modified live viral respiratory vaccine (IBRV-BVDV Type 1 and 2-PI3V-BRSV, Pyramid 5, Fort Dodge Animal Health, Overland Park, KS), a 7-way Clostridial bacterin/toxoid (Vision 7, Intervet/Schering-Plough Animal Health, DeSoto, KS), and dewormed with a moxidectin (Cydectin, Fort Dodge Animal Health, Overland Park, KS). All heifers were implanted with estradiol and trenbalone acetate (Component TE-G, Elanco Animl Health, Indianapolis, IN). Seven days after initial processing heifers were re-vaccinated with Pyramid 5 (Fort Dodge Animal Health).

Once moved to assigned pens heifers were offered prairie hay and 1% of BW of a diet consisting of (DM basis) 45% dry rolled corn, 15% corn WDGS, 17.5% ground alfalfa hay, 17.5% ground grass hay, and 5% pelleted supplement [69.6% wheat middlings, 2% limestone, 7.5% salt, 2% magnesium oxide, 0.3% zinc sulfate, 0.14% copper sulfate, 0.1% manganous oxide, 0.06% vitamin A (30,000 IU/g), 0.04% vitamin E (50%) and 0.25% Rumensin 80 (Elanco Animal Health, Indianapolis, IN)].

Heifers were observed daily for signs of BRD by trained professionals as described by Step et al. (2008). Briefly, evaluators used criteria based on the DART system (Pharmacia Upjohn Animal Health, Kalamazoo, MI) with modifications. The major observable signs were depression, appetite, respiratory signs, and rectal temperature. The heifers were assigned severity scores ranging from 1 to 4 where a 1 was assigned for mild symptoms, 2 for moderate, 3 for severe and 4 for moribund. The criterion for antimicrobial treatment was determined by using a combination of the visual observation ranking and rectal temperature. Animals that had a severity score of 1 and 2

had to have a rectal temperature of 40˚C or greater before an antimicrobial treatment was administered. If a heifer had a severity score of 3 or 4 she was automatically treated with an antimicrobial. The first antimicrobial treatment was tilmicosin (Micotil 300, Elanco Animal Health, Greenfield, IN) at a dosage rate of 10 mg/kg BW. If a second treatment was required (a minimum of 120 hours after first injection for severity scores of 1 or 2; 72 hours for 3 or 4), the antimicrobial was enrofloxacin (Baytril 100, Bayer Corp, Shawnee Mission, KS) at a dosage rate of 10 mg/kg BW. If a third treatment was needed (at least 48 hours after second treatment) ceftiofur hydrochloride (Excenel RTU, Elanco Animal Health) was used at a dosage rate of 2.2 mg/kg BW. Forty-eight hours after the third treatment heifers were given another treatment with ceftiofur HCl. When visual and temperature criteria were not met, no antimicrobial was given, and both treated and non-treated heifers were returned to home pens following evaluation. Body weight was recorded at the time of treatment and on d 63 to determine ADG during the preconditioning period.

After 63 days on feed, five heifers from the "healthy" (**HLTH**) group and five heifers from the "chronic" (**CHR**) group were chosen at random to be biopsied from the LM and SCF. Healthy was defined as an animal that had never been pulled for signs of BRD or any other disease and were never treated with an antimicrobial for clinical signs of BRD. Chronic was defined as heifers that had been identified as morbid and received antimicrobial treatment three times, were identified as being sick a fourth time, and lost BW during the previous 21 days. After the heifers were identified as CHR they were moved from the home pen to a chronic pen where heifers were placed on the growing diet

described above and given free-choice prairie hay for the duration of the receiving period (total 63 days).

On d 63, biopsies were taken from the SCF and LM. After using a local anesthetic and cutting through the skin, thumb forceps were used to raise the SCF and a sample (approximately 100 mg) was removed with scissors. Biopsies for LM tissue followed the procedure described by Winterholler et al. (2008). Biopsy samples were immediately snap frozen in liquid nitrogen, and stored frozen (-80˚C) until further processing.

RNA extraction

RNA was extracted from both tissues using TRIzol following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, ~0.2 g of tissue was homogenized in 1 mL of TRIzol. The homogenate was clarified by centrifuging at 12,000 × *g* for 10 min at 4˚C. This removed the excess fat, leaving a purer aqueous phase of RNA, DNA, and protein. Chloroform (0.2 mL) was added to the aqueous phase and incubated at room temperature for 10 min. Separation was done by centrifugation at 12,000 × *g* for 15 min at 4˚C. The aqueous phase (RNA) was removed and precipitated out with isopropanol. Ethanol (75%) was included to wash the pellet and then the pellets were dissolved in DEPC water.

A 1.5% agarose gel with ethidium bromide was used to determine quality of the RNA. The quantity was determined using a Nanodrop™ ND-100 spectrophotometer (NanoDrop Technologies, Willington, DE).

Quantitative Real-time PCR

Initially, a long oligo, whole bovine array from the Bovine Microarray Consortium (Elsik, 2006; http://animalsciences.missouri.edu/animalgenomics/bomc.php) was used to compare HLTH vs. CHR heifers (Johnson et al., 2009). Primers for genes of interest were designed using a sequence designed from Primer 3 (http://frodo.wi.mit.edu/) and Integrated DNA Technologies (IDT, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) software. The primer sequences, temperatures, and product size are shown in Table 1. Genes selected for real-time PCR were chosen based on their function. The broad categories were the ubiquitin-proteasome pathway [F-box and WD repeat containing 12 (**FBXW12**) and 26S proteasome (**26S**)], immunological functions [haptoglobin (**HP**), chemokine ligand 3 (**CXCL3**), and ribosomal protein S19 binding protein 1 (**RPS19BP1**)], acute phase factors (TLR-4, NF-κB, TNF-α, IL-6), and growth and metabolism [homeobox A5 (**HOXA5**), cadherin 1 (**CADH1**), and fatty acid binding protein 5 (**FABP5**)]. Total RNA (1 μg) was used to make cDNA with the QuantiTect® Reverse Transcription kit following the manufacturer's protocol (QIAGEN Inc., Valencia, CA). The cDNA was then used as the template for qRT-PCR.

An iCycler real-time detection system (Bio-Rad Laboratories, Inc., Herules, CA) was used along with PerfeCTa™ SYBR Green SuperMix for iQ™ (Quanta BioSciences, Inc. Gaithersburg, MD) to determine gene expression. 18S was used as the housekeeping gene. An efficiency curve for the reaction was done using five serial dilutions. Efficiencies ranged a total of six percentage points. Thermal cycling conditions were 95˚C for 30 s, followed by 40 cycles of 95˚C for 15 s, variable annealing temperatures for 30 s, and 72˚C for 30 s. This was followed by a melt curve analysis by increasing the

reaction to 95˚C for 1 min, 55˚C for 1 min, and then increasing the temperature by 0.5˚C from 55˚C to 94.5˚C. The samples were analyzed in triplicate.

Gene expression changes were calculated as described by Livak and Schmittgen (2001). A cycle threshold (C_T) was assigned to each of the samples. A ΔC_T value was calculated for each replicate by subtracting 18S rRNA C_T from the corresponding gene samples C_T. An average of the replicates was utilized to compare the gene expression levels. Fold change was calculated by determining the $\Delta\Delta C_T$. This was accomplished by subtracting expression levels for each treatment from the highest expression and plugged into the formula: fold change = $2^{-\Delta\Delta C}{}_T$.

Statistics

The experiment was a completely randomized design. Data were analyzed using the PROC MIXED procedure of SAS, and means were separated using the pdiff statement (SAS Inst., Inc., Cary, NC). Results are discussed as significant if $P \leq 0.05$ and as tendencies if $P > 0.05$ to $P \leq 0.10$.

RESULTS

Biological data

Biological data are shown in Table 2. From d 0 to 63, ADG (kg/d) generally decreased ($P < 0.001$) as the number of treatments for BRD increased.

Real time RT-PCR

In SCF, expression of FBXW12 tended ($P = 0.09$) to increase as the number of antimicrobial treatments increased (Figure 1). Fold change expression of FBXW12 was greater ($P < 0.01$) for T2, T3, and CHR compared with HLTH heifers; T1 were

intermediate. In addition, CHR heifers tended ($P = 0.06$) to have greater expression of FBXW12 in SCF than HLTH heifers (Figure 2). In LM, FBXW12 did not differ ($P = 0.39$) among treatments (Figure 3). However, when HLTH vs. CHR were compared, expression of FBXW12 tended ($P = 0.10$) to be greater in CHR than HLTH heifers (Figure 4). Similar to FBXW12, there was no difference ($P = 0.13$) in expression of 26S among treatments in LM (Figure 5). However, when HLTH vs. CHR were compared, expression of 26S tended to be greater ($P = 0.09$) in CHR compared with HLTH heifers (Figure 6).

Expression of NF-κB ($P = 0.49$) and TNF-α ($P = 0.92$) in SCF were not different among treatments (Figures 7 and 9). When comparing CHR vs. HLTH, NF-κB was not different ($P = 0.12$) *i*n SCF (Figure 8). However, in LM fold change expression of TNF-α was greater ($P = 0.01$) in CHR heifers than HLTH, T1 and T2; T3 heifers were intermediate (Figure 10). TNF-α was also greater ($P < 0.01$) in CHR heifers vs. HLTH heifers (Figure 11). Toll-like receptor-4 and IL-6 were not different ($P > 0.10$) among treatments in either tissue (data not shown).

Haptoglobin expression in SCF was greater ($P = 0.05$) in T1 than CHR heifers; HLTH, T2, and T3 heifers were intermediate (Figure 12). In the HTLH vs. CHR comparision HLTH heifers had greater ($P = 0.03$) expression of this gene than CHR heifers (Figure 13). Expression of other genes related to immune response (ITGB8, CXCL3, TRA1, and CATHL5) were not different ($P > 0.10$) among treatments.

DISCUSSION

Several factors play a role in the decreased performance observed in cattle treated for BRD. In the present experiment, we observed that with the increase in number of times an animal was treated for clinical signs of BRD there were increases in expression of components of the ubiquitin pathway in fat and muscle. Several experiments have suggested that the ubiquitin pathway, if over stimulated, can lead to chronic muscle wasting (reviewed by Cao et al., 2005). Specifically, atrophy of muscle occurs through activation of the ubiquitin-proteasome pathway. Proteins tagged with a combination of three different ubiquitins (E1, E2, and E3) are recognized and then degraded by the 26S proteasome. Expression of the E3 atrogin-1 has been shown to be eightfold greater in atrophied muscle from mice due to fasting (Gomes et al., 2001). The bovine equivalent of atrogin-1 is FBXW12. In the present experiment, the combination of this pathway along with the increase in TNF-α could explain the decreased ADG in CHR heifers. Tumor necrosis factor-α has been shown to activate muscle protein degradation via the ubiquitin and ATP-dependent proteolytic pathways (Garcia-Martinez et al., 1993). Tumor necrosis factor α in skeletal muscle of humans and animals with muscle wasting disease has been related to cachexia (Argilés et al., 2000).

Interestingly, in the present experiment FBXW12 was expressed in SCF with increases in the number of times a heifer was treated for clinical signs of BRD. The ubiquitin pathway is well documented to cause muscle wasting but the potential function of this pathway in SCF is not understood. A study by Cooke et al. (2007) used mice to look at F-box protein S kinase-associated protein 2 (**Skp2**), which is in the same family as FBXW12 and functions as an ubiquitone, to determine its effects on fat deposition.

Degradation of two cyclin-dependent kinase inhibitors involved in adipogenesis was activated by Skp2. Degradation of the inhibitors resulted in a decrease in subcutaneous fat due to both size and number of adipocytes (Cooke et al., 2007). Similar results in the bovine might explain the decrease in fat depots observed in cattle treated for BRD. Chibisa et al. (2008) showed that BW and fat loss in dairy cows were related to the up-regulation of the ubiquitin-proteasome pathway. Chibisa et al. (2008) fed propylene glycol to early lactation dairy cows and observed a tendency for decreased mRNA expression of several ubiquitin family genes in muscle.

In the present experiment, Hp gene expression was greater in SCF of HLTH and T1 heifers compared with CHR heifers. White adipose tissue is a primary source of HP mRNA, but brown adipose tissue can also express this gene (do Nascimento et al., 2004). An experiment conducted by do Nascimento et al. (2004) used 3T3-L1 adipocytes to determine changes in HP mRNA due to cytokines, catecholamines, and PPARγ. They observed that LPS, TNF-α, and IL-6 substantially increased HP mRNA. Dexamethasone, noradrenaline, isoprenaline, and β3-adrenoceptor agonist also increased expression of HP, while nicotinic acid and PPARγ agonist decreased mRNA expression of HP (do Nascimento et al., 2004). Haptoglobin is an acute phase protein and has been evaluated in several experiments as an indicator of inflammation and disease. In the present experiment, Hp was more highly expressed in HLTH heifers than in CHR heifers. Our results suggest that Hp may become less responsive with increased incidences of BRD.

In general, genes related with growth/metabolism (e.g., CADH1, FABP5) were not affected by treatment in the present experiment. Due to the established link between BRD and carcass merit, we hypothesized that differences would be observed. Perhaps

differences were negligible or missed due to the timing of tissue sampling in relation to the BRD events, or changes in genes related to the ubiquitin-proteasome pathway and immune function are mostly involved.

CONCLUSIONS

Genes involved in the ubiquitin-proteasome pathway, immunological function and the acute phase response were differentially expressed in the present experiment, especially for chronic heifers. These results may help explain observed differences in growth and carcass merit in cattle treated multiple times for BRD. Because our sampling protocol was at a single time point, a follow-up experiment may involve sampling at the time a heifer/steer is treated for clinical signs of BRD. Understanding gene expression changes at the time of treatment could potentially help in decreasing the detrimental effects of BRD early in the disease event.

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Table 1: RT-PCR primers

Gene	Tissue		Primer	Product Length	Anneal Temp.	Entrez ID
26S	LM	Forward	5'-CAGTGATGAAGGTGGAAAAGG-3'	184	59	506238
		Reverse	5'-GCGGACCATAGAGAATGACC-3'			
CADH1	SCF	Forward	5'-CGTTTCCTAAGTCGCTGGTC-3'	272	55.5	282637
		Reverse	5'-GGGCTTGTTGTCATTCTGGT-3'			
CATHL5	SCF	Forward	5'-AGGACGATGAGAACCCAAAC-3'	112	58	282167
		Reverse	5'-CCACACACTCTTTCAGCAGC-3'			
CXCL3	LM	Forward	5'-GCCAAACCGAAGTCATAGCC-3'	249	58	613667
		Reverse	5'-AAATAGTCCAGCACATCAAGTCC-3'			
FABP5	LM	Forward	5'-ATGGCTCTGCGAAAAGTGG-3'	145	59	281760
		Reverse	5'-GCTGTGGTCTCTTCAAACTTCTC-3'			
HOXA5	LM & SCF	Forward	5'-GACCTCGTTTAGTGCCAATG-3'	224	62-LM	768039
		Reverse	5'-CAGAGTCACAGTTTTCGTCACAG-3'		61-SCF	
HP	SCF	Forward	5'-CGTGTGGGTTATGTGTCTGG-3'	275	62	280692
		Reverse	5'-GTGTCGTCTTCCTTGTCGTG-3'			
ITGB8	SCF	Forward	5'-CAGTTTCACCATACATTAGCATCC-3'	244	58	3696
		Reverse	5'-AGCCTCTTTTCGCCATCC-3'			
RPS19BP1	LM & SCF	Forward	5'-CAGGAGAACCAGGAAGAAACC-3'	152	62-LM	509108
		Reverse	5'-TAGAACCCGAAGCCTACCC-3'		61-SCF	
TRA1	LM	Forward	5'-TCTTGCTGTGGTTTTGTTTG-3'	238	58	282646
		Reverse	5'-TTGTTCTTCCTCGTCTGTTCC-3'			
TLR-4	LM&SCF	Forward	5'-ACGAAAGCAGAAAGCCACAG-3'	265	59	281536
		Reverse	-GGTTACGGGAGGAGAGGAAG-3'			
NF-κB	LM&SCF	Forward	5'-GAGAGTGCTGGTGAAATGAGG-3'	287	58	540361
		Reverse	5'-TTTGCTGTTTGGTCTGTTGG-3'			
TNF-α	LM&SCF	Forward	5'-CCTCAGCCTCTTCTCCTTCC-3'	195	58	280943
		Reverse	5'-TCTTTCCCCATCAACACACC-3'			
IL-6	LM&SCF	Forward	5'-ACTTCTGCTTTCCCTACCCC-3'	233	57.5	280826
		Reverse	5'-CCGTCCTTTTCCTCCATTTT-3'			

Table 2: Biological data of heifers treated for BRD.

	Treatments[1]				
Item	HLTH	T1	T2	T3	CHR
ADG, kg/d[2]	1.52 ± 0.07	1.33 ± 0.08	0.87 ± 0.07	0.53 ± 0.06	0.61 ± 0.10
Days on feed					
1st treatment		16 ± 14	5 ± 2.1	3 ± 2.4	2 ± 1.5
2nd treatment			16 ± 4.7	12 ± 2.6	8 ± 2.2
3rd treatment				20 ± 3.9	13 ± 5.3
Days to removal[3]					32 ± 12.7

[1]Treatments were healthy (HLTH), treated once (T1), treated twice (T2), treated three times (T3), and chronic (CHR).

[2]ADG differed ($P < 0.001$) among treatments.

[3]Average day CHR heifers were moved to chronic pen.

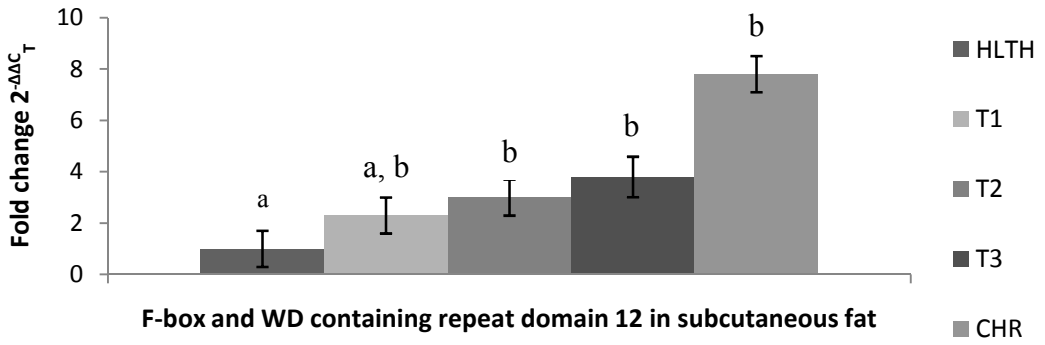

Figure 1: Fold change expression of FBXWD12 in SCF. There was a tendency for an increase in expression of FBXW12 with increases in the number of times and animal was treated for clinical signs of BRD ($P = 0.09$).
[a,b]Fold changes with different superscript letters differ ($P < 0.01$).

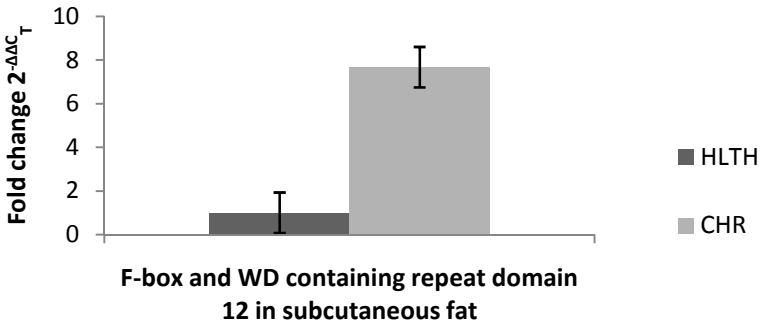

Figure 2: Fold change expression of FBXWD12 in SCF. Chronic heifers had a greater ($P = 0.06$) expression of FBXW12 than HLTH heifers.

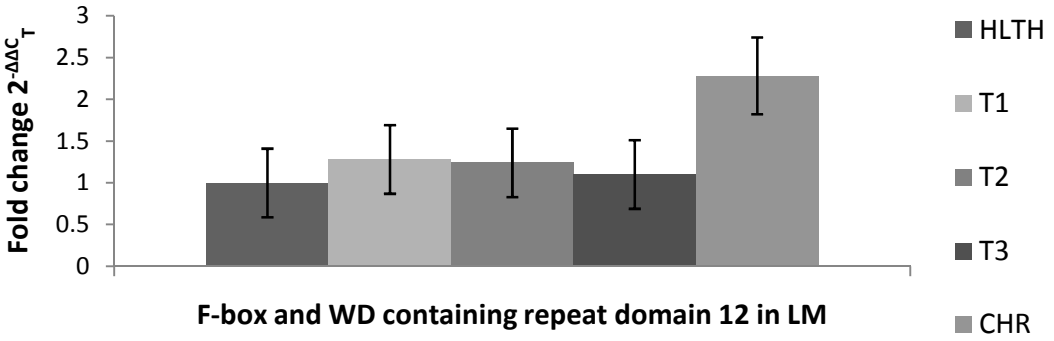

Figure 3: Fold change expression of FBXW12 in LM tissue. There was no difference among treatments ($P = 0.39$). Means separation indicated that CHR heifers tended ($P = 0.07$) to have greater expression than HTLH heifers.

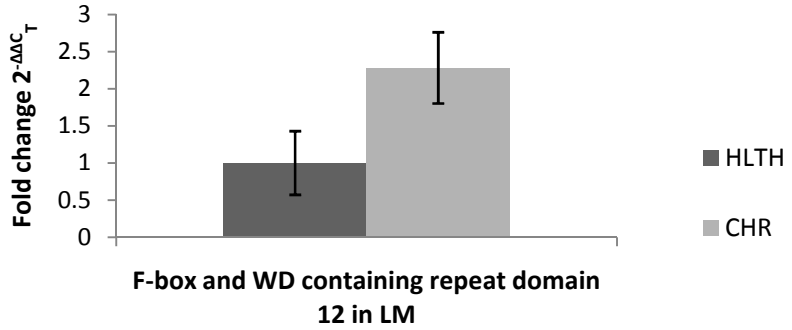

Figure 4: Fold change expression of FBXW12 in LM tissue. Gene expression tended ($P = 0.10$) to be increased for CHR heifers vs. HLTH.

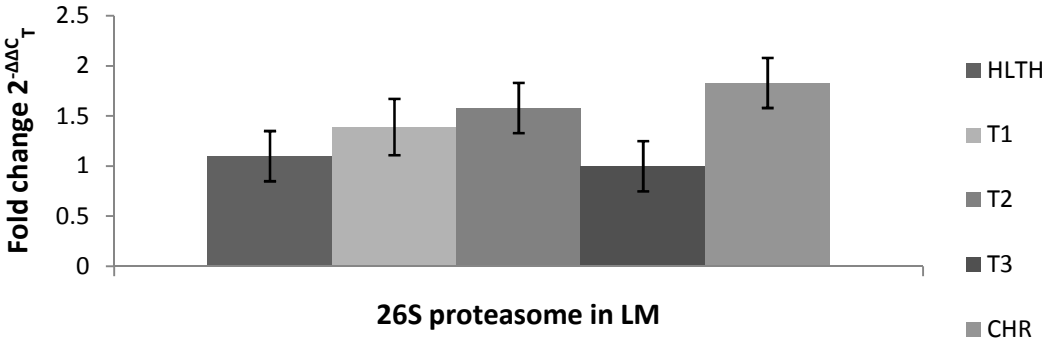

Figure 5: Fold change expression of 26S in LM tissue. There was no difference (P = 0.13) among treatments. Means separation showed that HLTH and T3 heifers had lower ($P \leq 0.05$) expression of 26S than T1, T2, and CHR heifers.

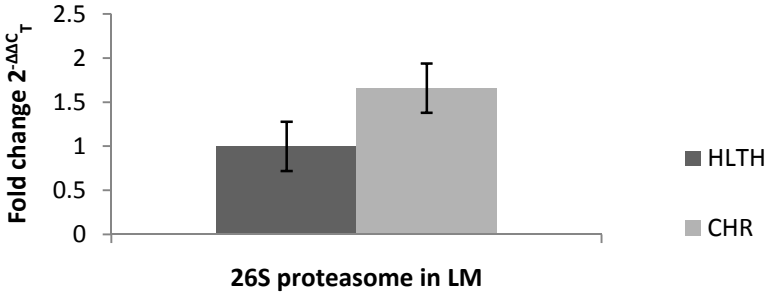

Figure 6: Healthy versus chronic fold change expression of 26S in LM tissue. 26S tended (P = 0.09) to be increased in the CHR heifer.

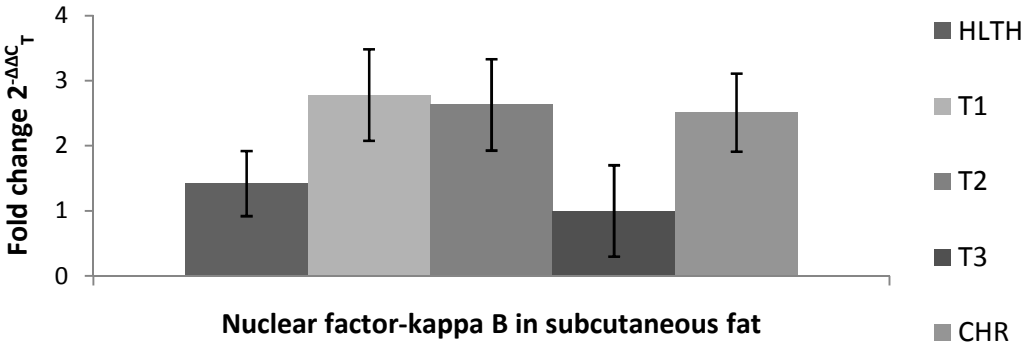

Figure 7: Fold change expression of NF-κB in SCF tissue. NF-κB was not different among treatments ($P = 0.49$).

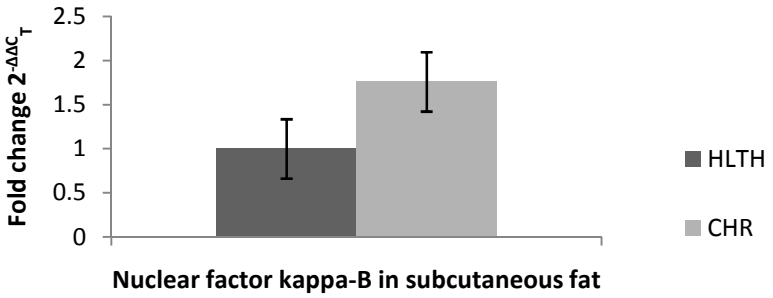

Figure 8: Healthy versus chronic fold change expression of NF-κB in SCF tissue. NF-κB tended ($P = 0.12$) to be increased in the CHR heifer.

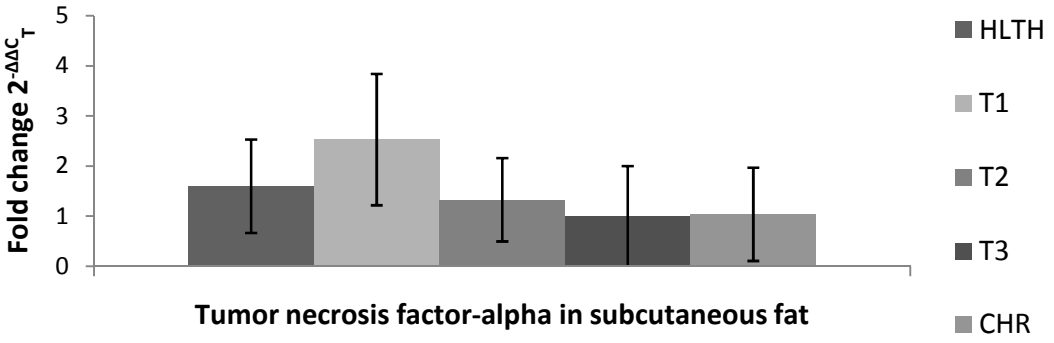

Figure 9: Fold change expression of tumor necrosis factor-α (TNF-α) within SCF tissue. Tumor necrosis factor-α was not different ($P = 0.92$) among treatments.

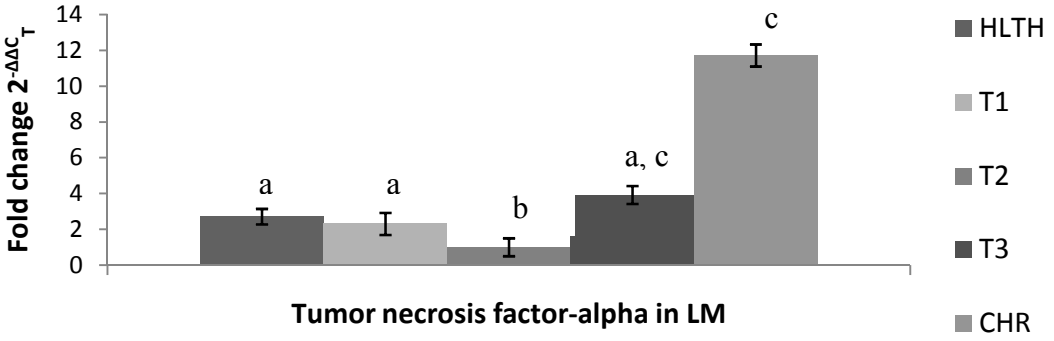

Figure 10: Fold change expression of tumor necrosis factor-α (TNF-α) in LM tissue. TNF-α was different among treatments ($P = 0.01$).
[a,b,c] Fold changes with different superscript letters differ ($P \leq 0.05$).

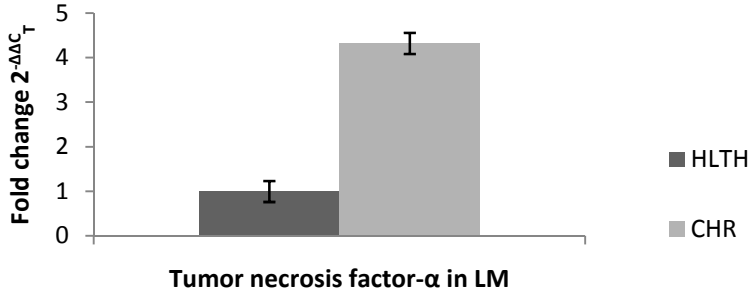

Figure 11: Healthy versus chronic fold change expression of tumor necrosis factor-α (TNF-α) in LM tissue. Tumor necrosis factor-α was greater ($P < 0.01$) CHR heifer than HLTH.

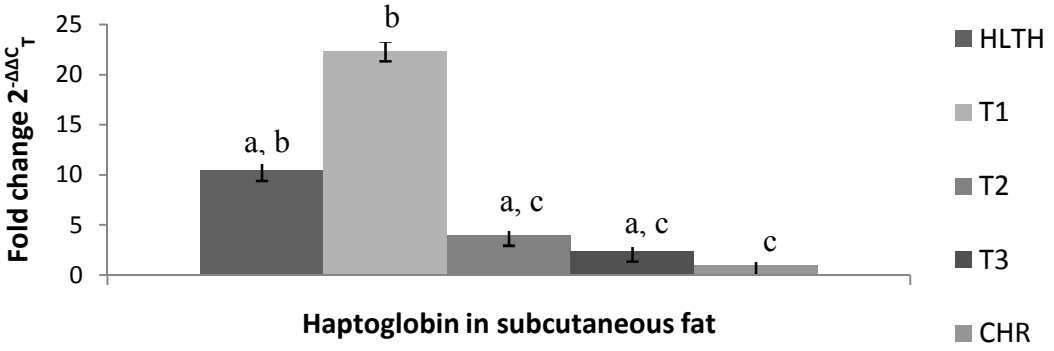

Figure 12: Fold change expression of haptoglobin (HP) in SCF. Haptoglobin was different among treatments ($P = 0.05$).
[a,b,c] Fold changes with different superscript letters differ ($P < 0.05$).

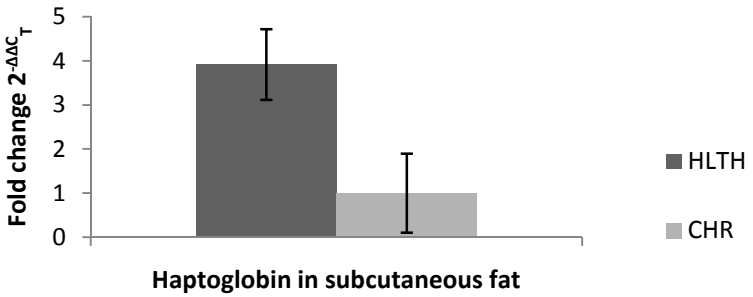

Figure 13: Healthy versus chronic fold change expression of haptoglobin (HP) in SCF tissue. Haptoglobin was greater ($P = 0.03$) HLTH heifers.

VITA

Jaymelynn Kay Johnson

Candidate for the Degree of

Master of Science

Thesis: GENE EXPRESSION CHANGES IN HEIFERS TREATED MULTIPLE TIMES FOR BOVINE RESPIRATORY DISEASE

Major Field: Animal Science – Immunology/Ruminant Nutrition

Biographical:

Personal Data:
Born and raised in Fort Sumner, NM. Graduated as Valedictorian in 2003.

Education:
Completed the requirements for the Master of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma in July, 2009.

Completed Bachelors of Science majoring in Animal Science – Pre-vet at Kansas State University, Manhattan, Kansas in May, 2007.

Completed Associates of Science in Agriculture at Butler Community College at El Dorado, Kansas in May, 2005.

Experience:
Cargill Animal Health – Pork production intern, 2007

Danbred North America – Pork production intern, 2006

Undergraduate laboratory assistant – Kansas State University growth and development lab, 2005-2007

Professional Memberships:
American Society of Animal Science
Honor Society

Name: Jaymelynn Kay Johnson Date of Degree: July, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: GENE EXPRESSION CHANGES IN HEIFERS TREATED MULTIPLE TIMES FOR BOVINE RESPIRATORY DISEASE

Pages in Study: 114 Candidate for the Degree of Master of Science

Major Field: Animal Science – Immunology/Ruminant Nutrition

Scope and Method of Study: Animal Science and Bovine Respiratory Disease

Findings and Conclusions:

Tissue biopsy samples from the longissimus dorsi muscle (LM) and subcutaneous fat (SCF) between the 12th and 13th rib from heifers never treated against BRD (HLTH; n = 5), treated once (T1), treated twice (T2), treated three times (T3), and heifers classified as chronically morbid (CHR; n = 5) were collected after a 63-d preconditioning period. Chronically morbid was defined as animals receiving at least three antimicrobial treatments and loss of BW during the previous 21 d on feed. Hybridizations were performed between CHR and HLTH using a long oligo bovine array. Significance level for differentially expressed genes was set at $P < 0.01$ with a twofold change or greater. Of the 186 differentially expressed genes in LM (143 down- and 43 up-regulated) and the 121 differentially expressed genes in SCF (44 down- and 77 up-regulated), 146 and 97, respectively, had known ontology. Differentially expressed genes were mapped to pathways involved in immunological functions, metabolism, catalytic activities, binding, proteolysis, apoptosis, translation, transcription, growth, and transport of nutrients. These differences in gene expression across tissues and between treatment groups will provide a better understanding of the impact BRD has on immune response and animal growth. In experiment two, genes (n = 14) were evaluated using quantitative RT-PCR. Proc Mixed procedures of SAS were used to evaluate significance of number of treatments. In SCF, components of the ubiquitin pathway, F-box and WD repeating domain containing 12 (FBXW12), tended to increase ($P \leq 0.10$) as the number of antimicrobial treatments increased. In addition, expression of FBXW12 tended ($P = 0.10$) to be greater and expression of 26S proteasome subunit ATPase 1 (26S) tended to be greater ($P = 0.09$) in LM of CHR heifers than HTLH heifers. In LM, fold change expression of tumor necrosis factor-α was greater ($P = 0.01$) in CHR heifers than HLTH, T1 and T2; T3 heifers were intermediate. Haptoglobin (HP) expression in SCF was greater ($P = 0.05$) in T1 than CHR heifers; HLTH, T2, and T3 heifers were intermediate. Results suggest that genes involved in muscle wasting via the ubiquitin pathways and expression of TNF-α may be activated in morbid heifers. These results could partially explain the decreased growth rates and carcass quality in cattle infected with BRD.

ADVISER'S APPROVAL: Dr. Clint Krehbiel